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Multisite Promiscuity in the Processing of Endogenous Substrates by Human Carboxylesterase 1

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Human carboxylesterase 1 (hCE1) is a drug and endobiotic-processing serine hydrolase that exhibits relatively broad substrate specificity. It has been implicated in a variety of endogenous cholesterol metabolism pathways including the following apparently disparate reactions: cholesterol ester hydrolysis (CEH), fatty acyl Coenzyme A hydrolysis (FACoAH), acyl-Coenzyme A:cholesterol acyltransfer (ACAT), and fatty acyl ethyl ester synthesis (FAEES). The structural basis for the ability of hCE1 to perform these catalytic actions involving large substrates and products has remained unclear. Here we present four crystal structures of the hCE1 glycoprotein in complexes with the following endogenous substrates or substrate analogues: Coenzyme A, the fatty acid palmitate, and the bile acids cholate and taurocholate. While the active site of hCE1 was known to be promiscuous and capable of interacting with a variety of chemically distinct ligands, these structures reveal that the enzyme contains two additional ligand-binding sites and that each site also exhibits relatively non-specific ligand-binding properties. Using this multisite promiscuity, hCE1 appears structurally capable of assembling several catalytic events depending, apparently, on the physiological state of the cellular environment. These results expand our understanding of enzyme promiscuity and indicate that, in the case of hCE1, multiple non-specific sites are employed to perform distinct catalytic actions.

Keywords: cholesterol metabolism; cholesterol esters; foam cells; atherosclerosis; heart diseases

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Abbreviations used: hCE1, human carboxylesterase 1; CoA, Coenzyme A; CE, cholesteryl ester; ACAT, acyl-Coenzyme A: acyl transferase; 4PP, 4-piperidino piperidine; FABP, fatty acid binding protein.

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Introduction

Several mammalian enzyme systems are involved in the processing and detoxification of both endobiotic and xenobiotic compounds. Central among these are the microsomal drug metabolizing enzymes, including the cytochrome P450s (CYP), the UDP-glucuronosyltransferases (UGT), and the non-specific serine hydrolases, including the human carboxylesterase isoforms 1 and 2. Each of these enzymes or enzyme families exhibits promiscuity in substrate recognition and acts on abundant, potentially toxic compounds from endogenous or exogenous sources. In many cases, the nature of enzymatic promiscuity remains mysterious, as it is not clear how one enzyme can act on structurally distinct substrates with acceptable levels of catalytic efficiency.

Human carboxylesterase 1 (hCE1) is a broad spectrum serine hydrolase involved in metabolizing a variety of clinical and elicit drugs, including cocaine, heroin, meperidine, lidocaine, ACE inhibitors, as well as organophosphate compounds.[1–9] The enzyme utilizes a standard two-step hydrolase mechanism in which the catalytic serine is activated for nucleophilic attack by adjacent histidine and glutamic acid residues in a catalytic triad. The acyl-enzyme intermediate formed between the serine and a portion of the substrate is released in the second step of the reaction either by an attacking water molecule (hydrolysis) or by an abundant alcohol (transesterification).[1,10] In addition to its role in drug and xenobiotic metabolism, hCE1 is implicated in processing a variety of endobiotics associated with cholesterol and fatty acid homeostasis.[1,3,4] The enzyme is expressed in numerous tissues associated with such protective roles, including liver, intestine, testis, kidney, lung, heart, and macrophage/monocytes.[1,11] hCE1 has been reported to catalyze cholesteryl ester hydrolysis (CEH) actions, in which a cholesterol-fatty acid conjugate is hydrolyzed to cholesterol and a free fatty acid (Figure 1(a)).[12,13] It has also been shown to perform a similar reaction with a fatty acyl Coenzyme A substrate, generating a free fatty acid and Coenzyme A.[14,15] The considerable size of each of these substrates suggests that the enzyme must contain two pores into its active site, which had been hypothesized for mammalian CEs[10] and is supported by the structural data reported here.

While the fatty acid acyl intermediate is covalently attached to hCE1, however, several transesterification reactions can occur rather than a simple hydrolysis, and these transesterifications are likely driven by the presence of an abundant alcohol-containing compound (Figure 1(a)). For example, hCE1 is known to act as a fatty acyl ethyl ester synthase, in which an ethanol is transesterified to a fatty acid. When free cholesterol is in abundance, hCE1 has been reported to catalyze acyl-Coenzyme A: acyl transferase (ACAT) action that generates cholesteryl esters.[16] Unraveling the structural bases of these varied catalytic actions is the focus of this article.

Monocytes and macrophages contribute to the development of atherosclerosis, the cause of coronary heart disease (CHD).[17] Fatty steaks, the primary atherosclerotic lesion, are initiated by the recruitment and differentiation of monocytes into macrophages at the endothelium of arterial walls. Macrophages then invade the arterial wall and take up significant quantities of oxidized LDL, forming excess cholesterol and fatty acids. These substrates then undergo re-esterification by acyl Coenzyme A cholesterol acyl transferase (ACAT) (Figure 1(a)). Excess cholesterol esters accumulate in cells, leading to the transformation of macrophages into foam cells forming atherosclerotic plaques, which can block coronary arteries causing a variety of coronary heart problems. hCE1, acting as a cholesteryl ester hydrolase (CEH), functions against ACAT by catalyzing the hydrolysis of cholesteryl esters into free cholesterol and fatty acids (Figure 1(a)).[12,13] Overexpression of hCE1 increases intracellular free cholesterol, essential in extracellular transportation, and prevents foam cell transformation.[12,13,17–20]

hCE1 homologs have also been shown to play a crucial role in fat and cholesterol metabolism and transportation. In mouse liver, when serum cholesterol is in excess (as in the case of ApoE knockout mice) the expression of mouse hepatic CE significantly increases.[21] In adipose tissues and intestine, these enzymes process the hydrolysis of triacylglycerol, the first step towards transporting free fatty acids and glycerol out of adipocytes *via* VLDL.[22–26] These enzymes also play an important role in the hydrolysis of long chain acyl CoA, such as palmitoyl CoA, in liver and kidney.[1,27]

Crystal structures of hCE1 have been reported in complexes with cocaine and heroin analogs, as well as with tacrine, mevastatin, tamoxifen and the potent dione inhibitor benzil.[10,28–30] These structures have revealed that the enzyme contains three ligand binding sites: the active site, which is located the bottom of a catalytic gorge, a "side door" secondary pore that leads into the active site from the surface of the enzyme, and the "Z-site" that controls the enzyme's trimer–hexamer equilibrium. The enzyme uses the Z-site surface to form a hexamer; thus, the Z-site is only available to bind to ligands when the enzyme is in its trimeric form.[28,29] While the role of the catalytic gorge in substrate binding is clear, the putative roles played by the side door and Z-site in shuttling substrates to the active site had not yet been firmly established structurally. Here, we examine the structural basis of hCE1's role in endobiotic metabolism. Four crystal structures are presented of hCE1 in complexes with Coenzyme A, the fatty acid palmitate, and the bile acids cholate and taurocholate (Figure 1(b)). Based on the various snapshots of ligand binding obtained from these structures, we present models for the manner in which hCE1 can act as a CEH, FACoAH, FAEEH and ACAT. These structures reveal that each of the ligand binding sites on hCE1 is promiscuous, and

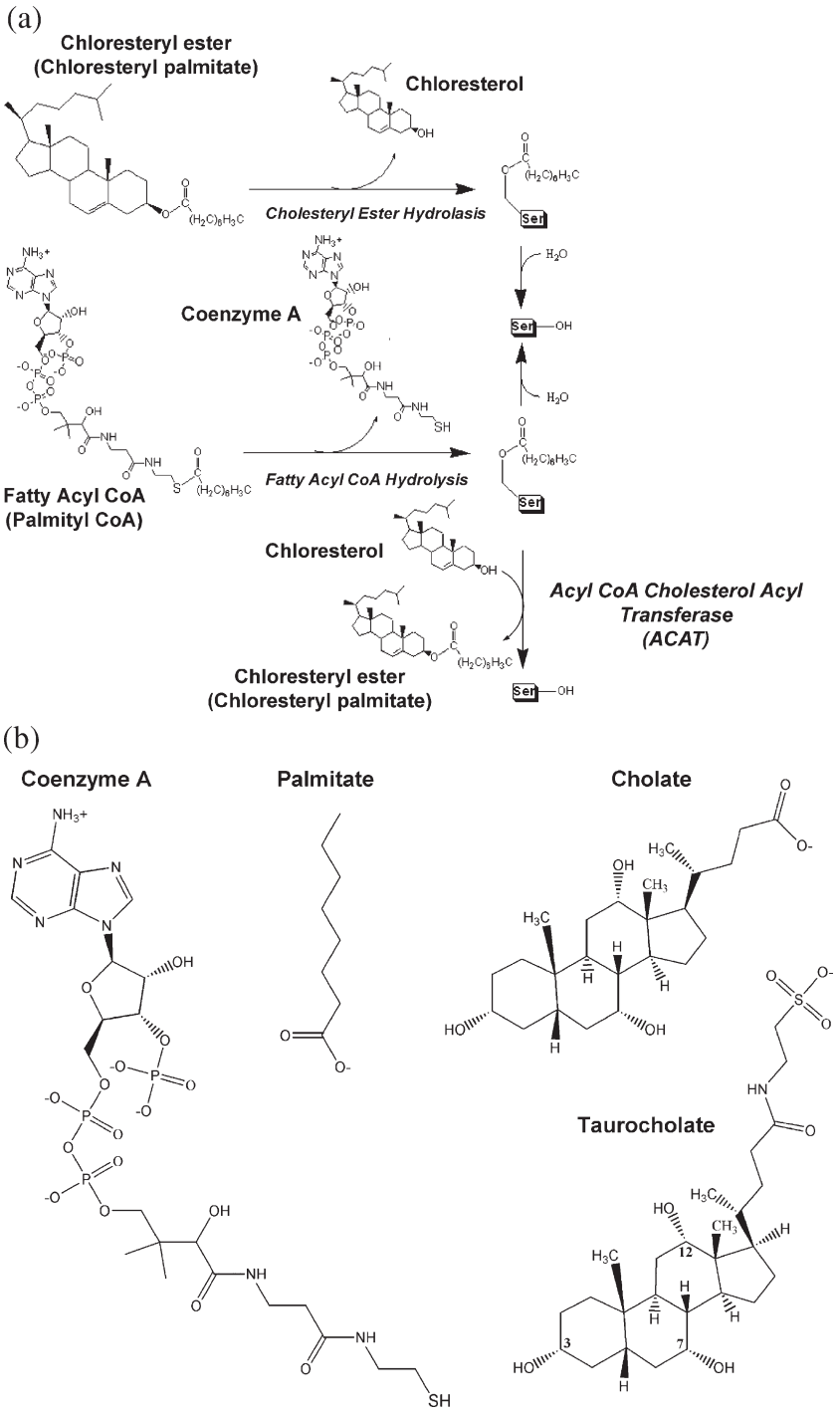

Figure 1. Endogenous substrate processing by hCE1. (a) Cholesteryl ester hydrolysis (CEH), fatty acyl CoA hydrolysis (FACoAH), and acyl CoA cholesterol acyl transferase (ACAT) reactions. (b) Endogenous ligands present in the crystal structures described here.

the enzyme appears to harness this promiscuity to assemble a variety of ostensively distinct catalytic functions.

Results

hCE1 structure

We have determined crystal structures of hCE1 in four complexes: CoA, homatropine/palmitate/CoA, palmitate/cholate, and taurocholate (Figure 1(b)). The cocaine analog homatropine is known from previous structural studies to stabilize hCE1 and was used as a co-crystallant in some cases to improve crystal quality. The 2.0 Å structure of hCE1-CoA complex is the highest resolution of hCE1 determined to date. The structures of hCE1-homatropine/palmitate/CoA, hCE1-palmitate/cholate and hCE1-taurocholate were refined to 2.8, 3.0 and 3.2 Å, respectively (Table 1). In one asymmetric unit, hCE1 forms a hexamer in complex with CoA, and a trimer in the remaining complexes.

The hCE1 monomer is formed by 17 α-helices and 20 β-strands and is divided into catalytic, αβ, and regulatory domains (Figure 2(a)). The catalytic

Table 1. Crystallographic statistics for human carboxylesterase 1 structures

	Coenzyme A	Homatropine/palmitate/ Coenzme A	Cholate/palmitate	Taurocholate
Resolution (Å; highest shell)	30–2.0 (2.0–2.13)	50–2.8 (3.0–2.8)	20–3.0 (3.2–3.0)	31–3.2 (3.4–3.2)
Space group	$P2_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Asymmetric unit	One hexamer	One trimer	One trimer	One trimer
Cell constants (Å, °)	a=88.99 b=115.37 c=175.53 β=90.05	a=55.56 b=181.02 c=202.56	a=55.29 b=179.88 c=201.32	a=55.42 b=179.95 c=201.09
Data collection facilities	SSRL	SSRL	UNC	SSRL
Total reflections	1,798,533	238,528	499,077	125,478
Unique reflections	233,023	50,837	38,284	34,194
Mean redundancy	7.7	4.7	13.0	3.7
R_{sym}[a] (%; highest shell)	8.2 (33.5)	12.3 (34.1)	11.0 (34.1)	14.4 (40.8)
Wilson B factor (Å^2)	21.6	48.4	47.8	45.6
Completeness (%; highest shell)	97.1 (88.1)	99.1 (99.7)	93.0 (83.6)	98.6 (97.6)
Mean I/σ (highest shell)	12.7 (2.9)	8.5 (2.8)	7.8 (2.4)	8.5 (3.0)
R_{cryst}[b] (%; highest shell)	18.4 (24.1)	19.3 (28.9)	22.6 (30.2)[d]	21.9 (28.6)[d]
R_{free}[c] (%; highest shell)	22.0 (27.4)	24.4 (35.5)	27.1 (35.3)[d]	25.5 (31.5)[d]
Number protein atoms[e]	24,726	12,390	12,390	12,390
Number solvent sites[e]	2715	508	269	253
Number carbohydrate atoms[e]	154	105	105	105
Number ligand atoms[e]	288	120	174	210
Number ion atoms[e]	60	33	30	30
Bound ligands[d]	One CoA in each active site	One palmitate in two active sites, one CoA in one active site; one homatropine in each Z site; one palmitate in side door	One palmitate in each active site; one cholate in each Z site	One taurocholate in each active site and each Z site

[a] $R_{sym}=\sum|I-\langle I\rangle|/\sum I$, where I is the observed intensity and $\langle I\rangle$ is the average intensity of multiple symmetry-related observations of that reflection.
[b] $R_{cryst}=\sum||F_{obs}|-|F_{calc}||/\sum|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively.
[c] $R_{free}=\sum||F_{obs}|-|F_{calc}||/\sum|F_{obs}|$ for 7% of the data not used at any stage of structural refinement.
[d] Non-crystallographic symmetry restraints were applied throughout the 3.0 Å and 3.2 Å trimeric complexes except areas (4–6 Å sphere) in contact with ligands.
[e] Number of atoms per asymmetric unit.

domain contains the catalytic triad composed of Ser221, His468 and Glu354, as well as a conserved high-mannose N-linked glycosylation found at Asn79. This carbohydrate moiety is thought to help in protein folding, solubility, and trimer stabilization.[29] The αβ domain provides the bulk of the buried surface area in the hCE1 trimer. The regulatory domain is composed of α10-12 and α16, and two novel Ω loops. This region of the enzyme exhibits relatively higher thermal displacement parameters (crystallographic *B*-factors), and has been proposed to regulate substrate binding and product release.[10,29]

hCE1 contains three ligand binding sites: the active site, the side door, and the Z-site (Figure 2(a)). The active site is located at the base of the catalytic gorge, which in hCE1 is relatively large and conformable to promote the promiscuous binding of substrates. Adjacent to the active site, and separated from the catalytic gorge by a thin wall of amino acid side-chains, is the side door. This ligand binding site, first identified in a related mammalian CE structure,[10] has been hypothesized to serve as a secondary substrate entrance and/or product release pore. The entrance to the catalytic gorge is flanked by α1 and α10′, a motif common to the active site of other fatty acid ester processing enzymes, including the palmitate protein thioesterases (PPT1 and PPT2).[31–33] Adjacent to the gorge opening, Ω1, Ω2, and α10′ form the Z-site,[28,29] a surface ligand binding site that controls the trimer–hexamer equilibrium of the enzyme.

hCE1-Coenzyme A complex

The crystal structure of hCE1 crystallized in the presence of Coenzyme A (CoA) was determined and refined to 2.0 Å resolution, the highest resolution of an hCE1 complex reported to date (Table 1). The enzyme packed into a monoclinic space group and is in its hexameric state in this complex, similar to the tacrine and naloxone structures determined previously.[28,29] Simulated-annealing omit electron density reveals the thiol tail of CoA bound adjacent to the hCE1 active site and present in two to three orientations per protein monomer (Figure 2(b)). We were unable to place the complete CoA molecule, however, which appeared to be mobile within the trimer–trimer interface of the hexamer. No ligand was observed bound at the side door region of the enzyme and, because of its hexameric state in this structure, the Z-sites of the enzyme were not available to interact with ligands. The position of the bound CoA tails suggests a mechanism by which the enzyme may hydrolyze the thioester linkages present in CoA-linked substrates, as discussed below.

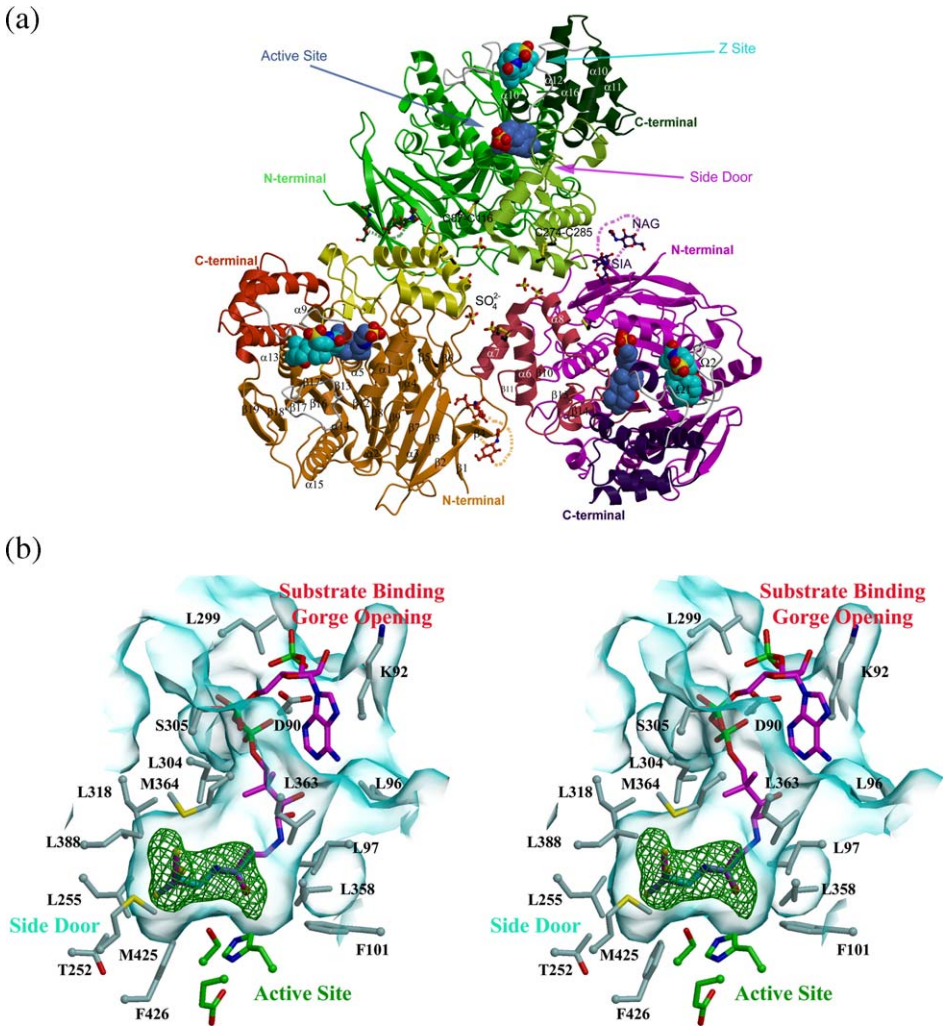

Figure 2. Overall structure and CoA binding. (a) The hCE1 trimer colored as follows: the catalytic domains are in green, magenta, and orange; the αβ domains are in light green, pink, and yellow; and the regulatory domains are in dark green, purple, and red. Taurocholate ligands are shown in cornflower blue in the active site and cyan in the Z-site (in between Ω1 and Ω2 loops shown in grey). The glycosylation modifications, disulfide linkages, and sulfate ions are also shown. (b) Stereoview of CoA within the active site of hCE1. Note that the thiol tail of CoA ligand is observed in three conformations. The surface of the substrate binding gorge is shown in cyan, while the surrounding residues are rendered in green for the active site and in grey for those lining the gorge. 2.0 Å resolution electron density contoured at 3σ from a simulated-annealing omit map calculated *via* $|F_{obs}|-|F_{calc}|$, ϕ_{calc} (with the ligand and a sphere 1.0 Å around the ligand omitted prior to annealing and map calculation) is shown in green.

hCE1-cholate-palmitate complex

We next co-crystallized the enzyme in complex with cholate (a bile salt and water-soluble cholesterol analog) and palmitoyl CoA, and determined the structure to 3.0 Å resolution (Table 1). In this case, the enzyme packed into an orthorhombic unit cell in its trimeric state, with one trimer per asymmetric unit. In structures of hCE1 determined previously, the enzyme was observed to shift from a hexamer to a trimer when its Z-sites contained bound ligand. Indeed, this complex revealed that cholate molecules were bound in all three Z-sites present in the trimer. The hydroxyl group at position 3 of cholate projects into the Z-site, forming a hydrogen bond with the carbonyl oxygen of Gly356. Intact palmitoyl CoA was used as a co-crystallant in this complex; thus, we were surprised to find upon inspection of the electron density at the active site of the enzyme that only palmitate, the product of palmitoyl CoA hydrolysis, appeared bound in each monomer of the trimer (Figure 3(a)). This observation suggests that the catalytic activity of the enzyme may have generated this product. In addition, as discussed below, this structure provides insights into the roles that endogenous ligand binding may play in enzyme function, as well as the orientational promiscuity of substrate binding at the enzyme's active site.

hCE1-palmitate-Coenzyme A-homatropine complex

The cocaine analog homatropine can be used as a co-crystallant to occupy the Z-site of hCE1, improving crystallization and diffraction quality.[29] Thus, we next co-crystallized hCE1 in the presence of both

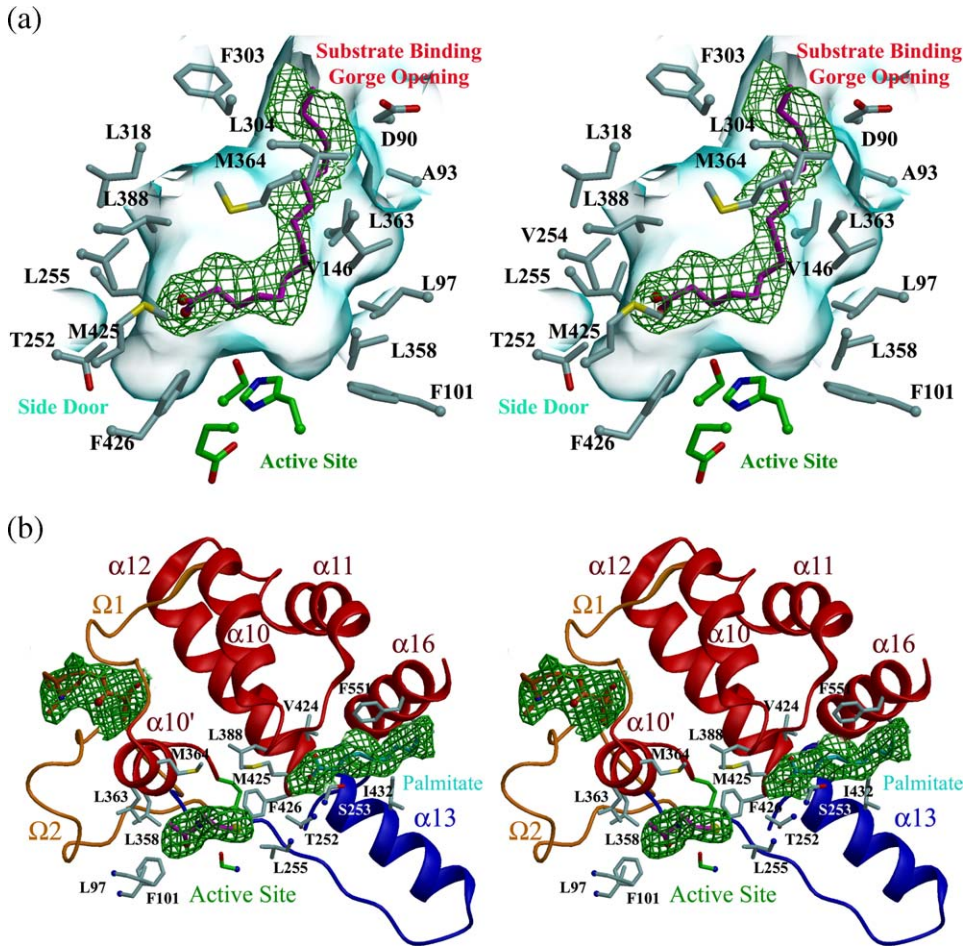

Figure 3. Fatty acyl CoA processing. (a) Stereoview of palmitate (magenta) within the substrate binding gorge of hCE1. Residues and surfaces are colored as shown in Figure 2(b). 3.0 Å resolution electron density contoured at 2σ from a simulated-annealing omit map calculated *via* $|F_{obs}|-|F_{calc}|$, ϕ_{calc} (with the ligand and a sphere 1.0 Å around the ligand omitted prior to annealing and map calculation) is shown in green. (b) Stereoview of palmitate (cyan) at the side door, the thiol tail of CoA (magenta) at the active site and homatropine (orange) at the Z-site. The catalytic and regulatory domains are in cornflower blue and red, respectively. The catalytic residues are shown in green, while some amino acid side-chains within the active site and side door are shown in light blue. 2.8 Å resolution electron density contoured at 2σ from a simulated-annealing omit map calculated *via* $|F_{obs}|-|F_{calc}|$, ϕ_{calc} (with the ligands and a sphere 1.0 Å around the ligands omitted prior to annealing and map calculation) is shown in green.

homatropine and palmitoyl CoA, and determined the structure of the complex to 2.8 Å resolution (Table 1). As expected, homatropine was found at each of the Z-sites in the trimeric asymmetric unit. Similar to hCE1-homatropine complexes previously described,[29] homatropine was observed to bind in two conformations at the Z-site of this complex and to form a combination of polar and non-polar contacts. At the active sites, we observed palmitate in two monomers of the asymmetric unit, bound in the orientation observed in the 3.0 Å resolution palmitate/cholate complex described above. In the third active site, we were surprised to find CoA bound as observed in the 2.0 Å resolution structure described above (Figure 3(b)). We were further surprised to find a palmitate molecule, contacted by Ser253, Val388, and Val424, bound at the side door of this particular monomer (Figure 3(b)). This is the first observation of a ligand bound to the side door of hCE1; to date, only 4-piperidino piperidine (4PP), a product of CPT-11 activation, has been observed bound to a mammalian CE side door, that of rabbit liver CE.[10] There are two features unique to this monomer of hCE1 that may explain why, of the three present in the asymmetric unit of this structure, only it exhibited binding to the CoA and palmitate products at the active site and side door, respectively. First, this monomer packs more tightly against other symmetrical molecules in the crystal lattice relative to the other two monomers. Thus, this more constrained environment may facilitate associations with two ligands. Second, the positions of two residues adjacent to the side door, Phe550 and Ser253, appear to accommodate the binding of the palmitate in this monomer relative to the other two (Figure 3(b)). The distance between these two residues in the palmitate-bound monomer is 6.8 Å, compared to 6.1 Å and 6.4 Å in the other two monomers of the trimer. The structure of this monomer advances our understanding of the potential for functional

interactions between the active site and side door in hCE1, as outlined below (see Discussion).

hCE1-taurocholate complex

Finally, to focus on the structural basis of cholesteryl ester hydrolysis by hCE1, we co-crystallized the enzyme with another water-soluble cholesterol analog, taurocholate, and determined the structure of this complex to 3.2 Å resolution (Table 1). The enzyme packs into an orthorhombic unit cell common to its trimeric form, with one trimer present per asymmetric unit. Taurocholate molecules are observed bound at each Z-site and active site within the trimer (Figure 4(a)). Similar to the cholate binding to the Z-site described above, the hydroxyl group at the 3 position of taurocholate forms a hydrogen bond with the main-chain carbonyl oxygen of Gly356 (Figure 4(b)). In addition to van der Waals contacts between the enzyme and taurocholate, the bile acid is further stabilized by water-mediated interactions between its polar groups at positions 7 and 12 and polar groups on hCE1. At the active site, the cholesterol scaffold of taurocholate is aligned such that the hydroxyl at its 3 position faces down into the substrate binding gorge and the flexible aliphatic sulfate tail unambiguously projects out towards the gorge opening (Figure 4(a)). Again, a series of polar and non-polar contacts is formed between the enzyme and ligand, including a salt-bridge between the sulfate group of the bile acid and the side-chain of Lys92 (Figure 4(b)). Note that the active site and Z-site of the enzyme are separated only by a single short helix, α10′ (Figure 4(b)). The structural features of this complex suggest how cholesterol-based substrates may be aligned for processing by the enzyme, and how the presence of ligands bound at the Z-site may impact catalytic actions within the substrate binding gorge of the enzyme.

Discussion

Z-site promiscuity and allostery

We have proposed previously based on structural and biophysical studies that ligand binding to the

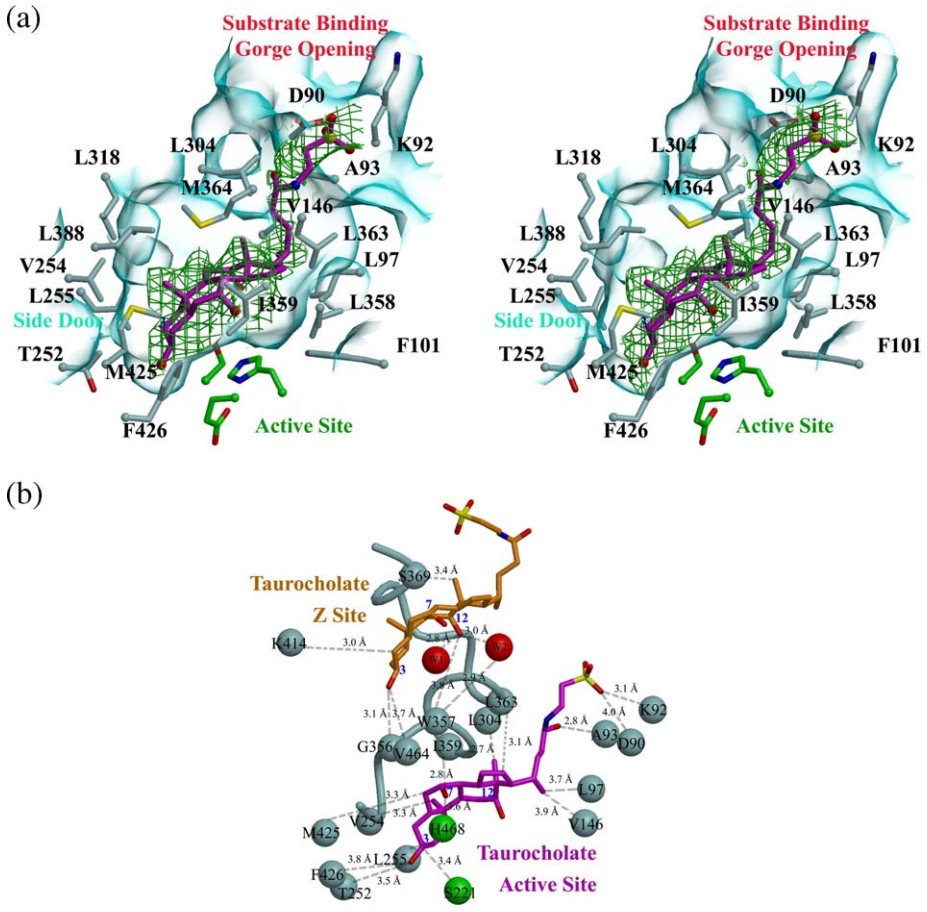

Figure 4. Taurocholate binding to hCE1. (a) Stereoview of taurocholate (magenta) within the substrate binding gorge of hCE1. Residues and surfaces are colored as shown in Figure 2(b). 3.2 Å resolution electron density contoured at 2σ from a simulated-annealing omit map calculated *via* $|F_{obs}|-|F_{calc}|$, ϕ_{calc} (with the ligand and a sphere 1.0 Å around the ligand omitted prior to annealing and map calculation) is shown in green. (b) Relationship between taurocholate bound at the active site (magenta) and Z-site (orange) in hCE1. α10′ separates the two sites, with contact residues shown as grey balls, the catalytic residues as green balls, and water molecules in the Z-site as red balls.

hCE1 Z-site shifts the enzyme's trimer–hexamer equilibrium toward trimer, facilitating the binding of substrates within the active site and promoting catalysis.[29,30] The data presented here support this hypothesis. In addition, they further suggest that the Z-site is capable of binding to cholesterol-like molecules, a feature that may play an important role the biological activity of the enzyme in terms of processing cholesteryl esters and other endogenous substrates. The binding of cholesterol-scaffold ligands to the hCE1 Z-site would shift the enzyme from its hexamer to its trimer state, opening up access to its active site and, presumably, promoting catalysis. The Z-site may also play a more direct role in the allosteric activation of catalysis. The catalytic triad residue Glu354 lies on a turn just prior in sequence to α10′, the helix that physically separates the active site and Z-site regions of hCE1 and is directly contacted by ligands bound in both sites (Figure 4(b)). The association of compounds at the Z-site may facilitate the proper positioning of Glu354 for catalysis. Indeed, in the structure of the related rabbit liver CE, in which the region corresponding to α10′ was disordered, the Glu354-equivalent residue (Glu353) was observed in a position not amenable to catalysis.[10]

These structural observations, as well as recent kinetic data indicating that inhibitors that associate with both the active site and Z-site are mixed-type inhibitors of hCE1,[30] strongly suggest that the Z-site functions allosterically in hCE1 activity. In addition, the wide variety of compounds observed to bind to the hCE1 Z-site (e.g. homatropine, tamoxifen, cholate, taurocholate) firmly establish the promiscuity of this region of the enzyme, a feature that mirrors the promiscuity of the active site. If the Z-site functioned allosterically, such relative non-specificity in ligand binding would be expected. Recently, the crystal structure of the human cytochrome P450 CYP3A4 isoform revealed that, similar to the Z-site in hCE1, this drug metabolizing enzyme contains a surface ligand binding site adjacent to its active site.[34] This peripheral binding site is proposed to function in substrate screening and allosteric regulation.[34] A similar role may exist for the Z-site of hCE1, a conclusion that is supported by the data presented here and by previous structural and functional studies.[29,30]

hCE1 side door structure and function

While a product of CPT-11 activation 4PP had previously been observed bound at the side door region of rabbit liver CE (rCE),[10] the observation of the fatty acid palmitate bound at hCE1's side door is the first for this human CE isoform. There are three common features of the side door shown in the crystal structures of hCE1, rCE and a related enzyme, bovine bile salt activated lipase (bBAL).[10,35,36] These include (1) a "gate" residue (Met425, Leu424, and Leu392 in hCE1, rCE and bBAL, respectively); (2) a conserved "switch" Phe (Phe426, Phe425, and Phe393 in hCE1, rCE and bBAL, respectively) located in between α12 and α13; and (3) an aromatic "releasing value" residue (Phe551, Trp550, and Tyr526 in hCE1, rCE and bBAL, respectively) located in the terminal helix of each enzyme (Figure 5(a) and (b)).

First, the gate residues are highly variable and mutations of these residues alter the activities of the enzyme towards particular substrates.[37,38] The binding of palmitate at the side door in the hCE1 structure suggests that Met425 is perhaps the most important residue in the release of the fatty acid after hydrolysis of cholesteryl esters or acyl CoA. This residue corresponds to that described by Wallace *et al.*,[37] in which a mutation of the equivalent residue in a rat homolog of hCE1 alters its substrate preference from carboxylesterase to cholesterol ester hydrolase. In addition, the structures of bBAL suggested that the fatty acid leaving product may have exited the enzyme through a hydrophobic channel analogous to the side door that is guarded by only one tryptophan residue, Trp227.[35,36]

Second, we have observed a conserved Phe between two connected helices of the catalytic domain and the regulatory domain, immediately after Met425. This residue provides a link between the catalytic and the regulatory domains. Therefore, we hypothesize that when the product exits the active site through the gate residues, it would induce a conformational change in the area of these helices. We propose that the conserved Phe functions as a

Figure 5. Mechanistic hypotheses for endogenous substrate processing. (a) The crystal structures of the hCE1-taurocholate complex, the bovine salt-activated lipase (bCE)-taurocholate complex, and the rabbit liver carboxylesterase (rCE)-4-piperidinopiperidine (4PP) complex are shown. The three domains of hCE1 and rCE (catalytic, αβ, and regulatory) are rendered in cornflower blue, green, and red, respectively, while the lipase is shown in magenta. Note that the taurocholate surface binding sites of hCE1 (the Z-site) and the lipase are distinct. (b) The active site and side door regions of hCE1, rCE and the bovine salt-activated lipase (bBAL). Note that the ligands, palmitate (in cyan) for hCE1, 4PP (in purple) for rCE, and taurocholate (in orange) for bBAL, are located at an equivalent side door region in each enzyme located in proximity to the active site. (c) Schematic models of fatty acyl CoA hydrolysis and fatty acyl ethyl ester synthesis by the hCE1 hexamer. When the fatty acyl CoA is abundant, it is possible that the hexameric hCE1 would allow the acyl CoA substrate to enter the active site through the side door. See (d) for a definition of the schematic regions of hCE1. (d) Schematic models of cholesteryl ester hydrolysis, fatty acyl CoA hydrolysis, and acyl CoA cholesterol acyl transferase by the hCE1 trimer. First, excess cholesterol could bind the Z-site and shift the trimer–hexamer equilibrium towards trimer. Second, the substrates fatty acyl CoA or a cholesteryl ester could enter the active site through the main substrate binding gorge. For hydrolysis reactions, products (e.g. CoA, cholesterol and fatty acids) could leave through the main substrate binding gorge and the side door. For a transesterification, cholesterol may enter the active site *via* the substrate binding gorge, producing a cholesteryl ester.

hinge allowing rotation of the regulatory domain, while Met425 functions as a gate allowing the release of product such as fatty acids through the side door.

Finally, the conserved aromatic residue in the terminal helix is always seen in association with the product at the side door. It has been proposed that the binding of taurocholate at the equivalent side door in bBAL may help to organize this region of the enzyme for the release of long chain fatty acids.[36] In rCE, this stabilization of the side door is provided by a carbohydrate chain that results from post-translational modification of the protein;[10] this glycosylation site is missing in hCE1. We hypothesize that this aromatic side-chain along with other additional functional domains (e.g. carbohydrate modifications or bile salts) may provide a valve to allow release of products from the side door. In addition, it may function as a secondary signaling

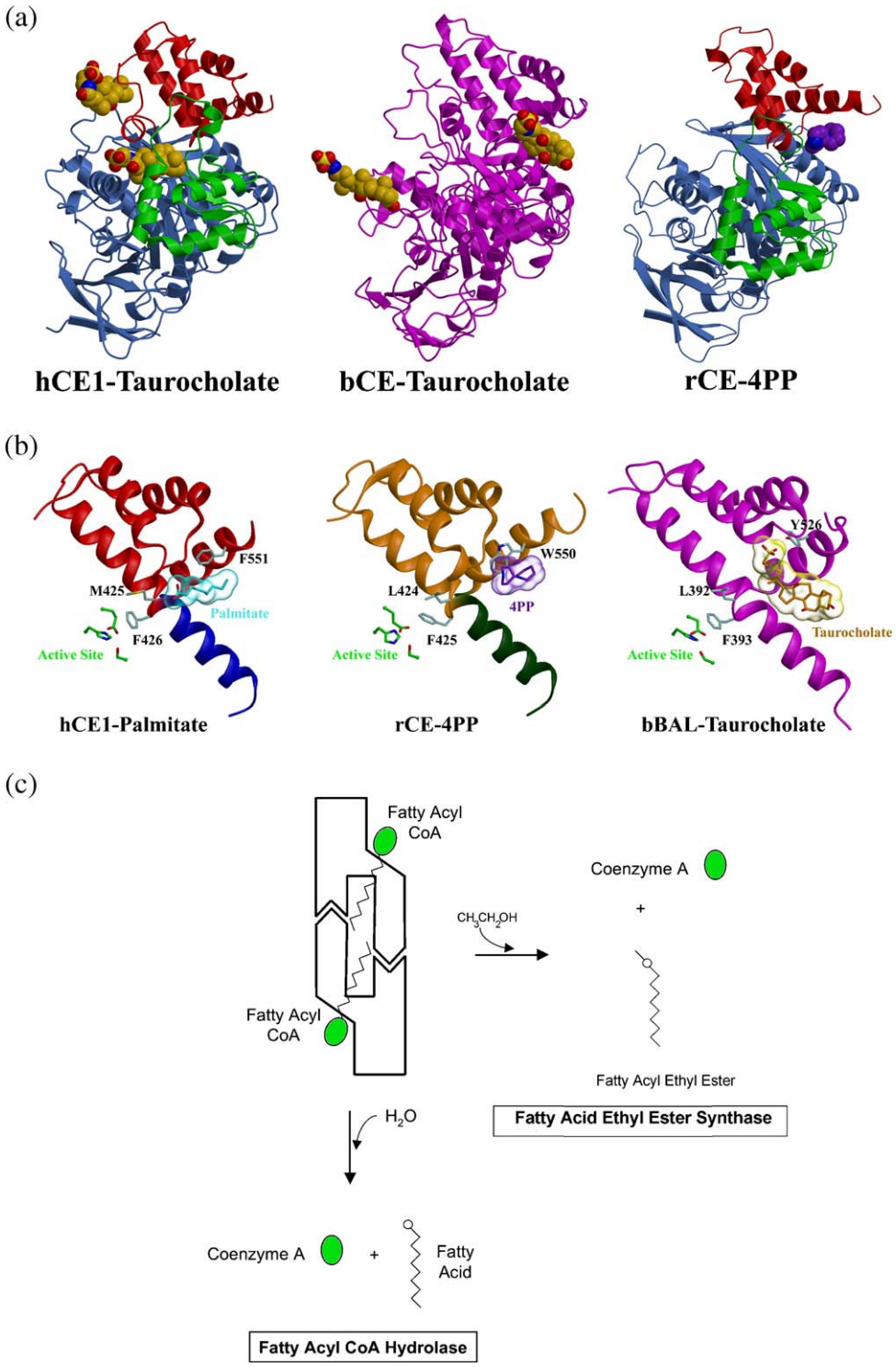

Figure 5 (*legend on previous page*)

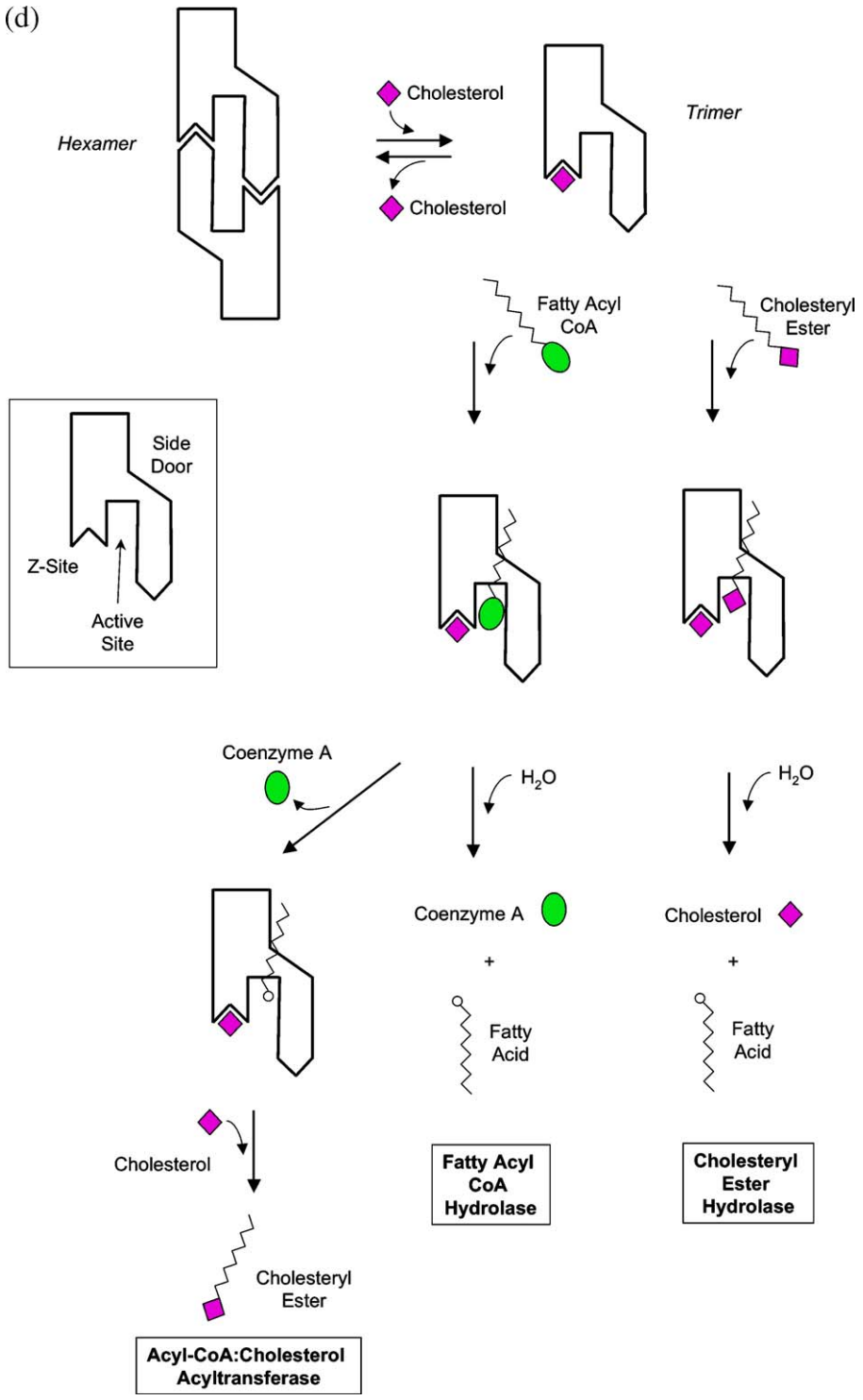

Figure 5 (*legend on page 208*)

mechanism for the regulatory domain, and may allow the release of ligands both at the active site and the Z-site.

Catalytic promiscuity toward endogenous substrates

The four structures presented here suggest a variety of sometimes conflicting models for the processing of large, endogenous substrates by hCE1. For example, the hCE1-CoA complex indicates that cleavage of a fatty acyl-CoA substrate would require that the long fatty acyl tail extrude from the active site of the enzyme through the side door to allow for the positioning of the scissile thioester linkage at the catalytic residues. In contrast, the orientation of palmitate product observed in the hCE1-cholate-palmitate structure,

in which palmitate was apparently generated *via* cleavage of the palmitoyl CoA compound used in crystallization, suggests the opposite orientation with the CoA at the side door and the palmitate in the catalytic gorge. The static nature and structural features of these two complexes do not allow us to distinguish between these options. However, the snapshot provided by one monomer of the three present in the hCE1-palmitate-CoA-homatropine complex lends support to the mechanism in which a fatty acyl-CoA binds with the CoA in the catalytic gorge and the fatty acid tail extruded *via* the side door. In this complex, CoA and palmitate were observed bound at the active site and side door regions of the enzyme, respectively, which are the positions expected if the substrate utilized extrusion of the fatty acid tail from the side door. This orientation also suggests that the palmitate product would be released from the catalytic gorge *via* the side door, as had been proposed previously for 4PP in the rabbit liver CE.[10] In terms of cholesteryl ester hydrolysis, the taurocholate-bound hCE1 structure also suggests that productive hydrolysis would require extrusion of the fatty acyl tail from the active site *via* the side door.

Based on the structural data presented here, two overall scenarios of hCE1 action in the processing of endogenous substrates can be envisioned (Figure 5(c) and (d)). In the first scenario, hCE1 is considered in its hexameric state (Figure 5(c)), in which the binding of a ligand or substrate to the Z-site has not shifted the trimer–hexamer equilibrium toward trimer. For large aliphatic substrates like fatty acyl Coenzyme A, access to the active site of hCE1 may be gained through the side door, which would align the thioester linkage for catalysis with the larger Coenzyme A moiety located outside the enzyme. This arrangement would allow for either hydrolysis for fatty acyl CoA hydrolase activity or for transesterification for fatty acid ethyl ester synthase activity (Figure 5(c)). The binding of the palmitate fatty acid with its charged carboxylate adjacent to the catalytic residues in palmitate/cholate structure described here (Figure 3(a)) is consistent with this model. Recall that palmitoyl-CoA was used for co-crystallization in this complex so the palmitate observed was likely generated by fatty acyl CoA hydrolysis.

In the second scenario, hCE1 is considered in its trimeric state, in which the binding of a ligand (e.g. cholesterol as shown schematically) has shifted the trimer–hexamer equilibrium toward trimer (Figure 5(d)). More open access to the catalytic gorge of the enzyme allows substrates to bind directly to the active site. For both fatty acyl CoA and cholesteryl esters, however, productive binding would require the extrusion of the fatty acyl tail out of the active site through the side door, as shown schematically (Figure 5(d)). The observed binding of palmitate at the side door in the homatropine/palmitate/CoA structure (Figure 3(b)) and of taurocholate at the active site in the taurocholate structure (Figure 4(a) and (b)) are consistent with this proposal, as are the observed binding of the cholate and taurocholate in the Z-sites of the palmitate/cholate, and taurocholate structures, respectively (Figures 2(a) and 4(b)). Such arrangements allow for the enzyme to act as a cholesteryl ester hydrolase, and fatty acyl CoA hydrolase or, *via* transesterification with cholesterol, as an acyl-CoA:cholesterol acyltransferase (ACAT; Figure 5(d)). Other enzymes that specifically catalyze palmitoyl ester hydrolysis including palmitoyl-protein thioesterases (PPTs) have obvious alcohol and acyl binding sites.[31,39] All PPT structures show a specific binding groove for palmitate. However, our structures suggest that hCE1 has no rigidly assigned acyl or alcohol binding sites, but rather orients the substrate promiscuously. The promiscuous binding of fatty acid substrates has not been previously reported and may be unique to hCE1 and hCE1-like enzymes. It has been shown that in a rat liver trimeric homolog of hCE1 functions as fatty acid ethyl ester synthase and specific inhibitors can be used in cell culture to prevent the formation of fatty acyl ethyl esters (FAEE).[40,41]

One dilemma with this model for hCE1 catalysis in its trimeric state is that the largely hydrophobic fatty acid leaving group would be released into the cytoplasm. We propose that this fatty acid product be bound by a lipid cargo protein such as the fatty acid binding protein (FABP). There is evidence that hormone-sensitive lipase (HSL), another cholesterol ester hydrolase, functions together with FABP. Tissue-specific FABP will bind to HSL only when there are fatty acids present, with the absence of fatty acids completely abolishing this interaction.[42] FABPs are present in all tissues that express hCE1, and thus FABP may facilitate the departure of the free fatty acid product through the side door.

It is important to note that cholesterol is shown schematically bound to the Z-site of hCE1 in Figure 5(d); however, one can envision other ligands, substrates, or substrate analogs bound in that putative allosteric site to shift the enzyme's oligomeric state toward its more open trimer form. In addition, one can also imagine the hCE1 trimer floating on a lipid droplet with its active sites facing "down"[19,20] and in this way able to draw cholesteryl ester substrates directly into its active site. Thus, the enzyme's multiple sites (active, Z, side door) and oligomeric state (trimer, hexamer) may respond in various ways to the physiological nature of the cellular environment. During foam cell formation or reverse cholesterol transport, hCE1 may interact with lipid droplets as described and process abundant substrates as a cholesteryl ester hydrolase, for example. Under other conditions, the enzyme may reverse its action and perform fatty acyl CoA hydrolysis or ACAT events (Figure 5(d)). In all cases, it would be expected that hCE1 acts as an important back-up to other enzyme systems charged with the primary tasks of processing endogenous substrates (e.g. the human ACAT

isoforms), and is called into action when abundant compounds are present. Thus, the utilization of an enzyme that contains multiple promiscuous sites and the ability to bind to substrates in various orientations would be expected to be a great asset to cell types like hepatocytes and macrophages that must adjust to significant changes in the levels of key endogenous substrates.

Experimental Procedures

Crystallization

A secreted, 62 kDa form of hCE1 was expressed using baculovirus in *Spodoptera frugiperda* Sf21 cells and purified as described.[43,44] hCE1 was concentrated to 3 mg ml^{-1} in 50 mM Hepes (pH 7.4), and crystallized in the presence of different ligands; 10 mM Coenzyme A, 10 mM homatropine-10 mM palmitoyl Coenzyme A, 10 mM cholic acid-10 mM palmitoyl Coenzyme A, or 10 mM taurocholate; using sitting-drop vapor diffusion at 22 °C. Crystals of 200–300 μm in size grew in 8% (w/v) PEG-3350, 0.4 M Li_2SO_4, 0.1 M NaCl, 0.1 M LiCl, 0.1 M citrate (pH 5.5), 5% (v/v) glycerol. In the case of homatropine-palmitoyl Coenzyme A complexes, 0.1 mM NaF was also added. The crystals were cryo-protected in 20–40% (w/v) sucrose plus 75–50% (v/v) mother liquor prior to flash cooling in liquid nitrogen.

Structure determination and refinement

Diffraction data were collected at Stanford Synchrotron Radiation Laboratory (SSRL) beamlines 9-1 and 9-2, and at the X-ray Facility at the University of North Carolina-Chapel Hill. The experiments were performed at 100 K using cryo-cooled crystals, and were processed and reduced using DENZO and SCALEPACK, MOSFLM, and HKL2000.[45,46] These hCE1 structures were determined by molecular replacement (AMoRe)[47] using the structure of hCE1 in complex with tacrine (PDB code: 1MX1)[28] as a search model. Residues 21–553 of the 566 amino acid enzyme were traced for each protein monomer. Structures were refined using torsion angle dynamics in CNS[48] with the maximum likelihood function target, and included an overall anisotropic *B*-factor and a bulk solvent correction. Representative 5–7% of the observed data were set aside for cross-validation using free-*R* prior to any refinement. In the higher resolution structures, hCE1-CoA (2 Å) and hCE1-homatropine-palmitate-CoA (2.8 Å) complexes, non-crystallographic symmetry (NCS) restraints were employed for each structure at initial refinement stages, and then removed such that each monomer was refined independently. However, for the moderate resolution structures (3.0–3.2 Å) we utilized total NCS restraints in the initial refinement stages and later kept the NCS restraints only in the residues that have no direct contact with the ligands (at least 4 to 6 Å away from the ligands). Manual adjustments were performed using the program O[49] and σ_A-weighted[50] electron density maps. Simulated annealing omit and σ_A-weighted difference density maps were used to position ligands into electron density at the active site, Z-site, or side door. N-linked glycosylation sites were traced in all complexes. Final structures exhibit good geometry with no Ramachandran outliers. Molecular graphic Figures were created with MolScript,[51] BobScript,[52] Raster3D,[53] Grasp,[54] and Dino†.

Protein Data Bank accession codes

Atomic coordinates have been deposited with the RCSB Protein Data Bank and are available under accession codes 2H7C, 2DQZ, 2DQY, and 2DQ0.

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