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Delivering Products**

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Enzymes Without Borders: Mobilizing Substrates, Delivering Products

Federico Forneris and Andrea Mattevi*

Many cellular reactions involve both hydrophobic and hydrophilic molecules that reside within the chemically distinct environments defined by the phospholipid-based membranes and the aqueous lumens of cytoplasm and organelles. Enzymes performing this type of reaction are required to access a lipophilic substrate located in the membranes and to catalyze its reaction with a polar, water-soluble compound. Here, we explore the different binding strategies and chemical tricks that enzymes have developed to overcome this problem. These reactions can be catalyzed by integral membrane proteins that channel a hydrophilic molecule into their active site, as well as by water-soluble enzymes that are able to capture a lipophilic substrate from the phospholipid bilayer. Many chemical and biological aspects of this type of enzymology remain to be investigated and will require the integration of protein chemistry with membrane biology.

The hallmark of cell architecture is the organization in compartments and organelles that are separated by phospholipid membranes. This property intrinsically defines two main cellular environments with substantially different physical-chemical properties. Hydrophobic compounds tend to partition preferentially into lipid bilayers, whereas the aqueous environments of the cytosol and the interiors of cellular organelles create a favorable medium for polar substances (1). However, between the hydrophobic membranes and the aqueous intra-organelle environment, there is not a static and rigid separation. Rather, there is a continuous reshuffling of metabolites, as well as a traffic of macromolecules (2–4), and many cell processes involve both hydrophobic and hydrophilic substrates—i.e., substances that typically belong to the membrane phase and water phase of the cell, respectively.

Enzymatic reactions involving compounds that reside within organelle lumens and the membranes have peculiar mechanistic requirements. If the enzyme is located in a water-soluble environment, how is the active site made accessible to hydrophobic substrates? Conversely, for a membrane-embedded active site, what are the mechanistic requirements for admission of hydrophilic substrates? An important concept that needs to be clarified is the definition of hydrophobic and hydrophilic substances (5). Considering the chemical diversity of the compounds that populate the cell compartments, it is impossible to unambiguously classify each of these molecules as “hydrophobic” or “hydrophilic.” In general, we will define as “hydrophilic” the water-soluble compounds that are commonly present in the aqueous lumen of cell organelles, and as “hydrophobic” the molecules that belong

to the lipid bilayer, even though they may have limited solubility in water solutions.

We have conducted a survey of the three-dimensional protein structures deposited in the Protein Data Bank (6), which has led us to identify a set of enzymes that can provide a structural framework to the reactions involving substrates of opposite chemical nature. This survey is clearly not intended to be a comprehensive analysis, which would be beyond the scope of this review. Our aim is to illustrate the mechanistic strategies underlying this type of cellular reaction and to frame a context for further research that is likely to require the development of new methods and concepts in enzymology.

Water Reacting with Lipophilic Substrates

An elegant example of the use of a hydrophilic compound in a hydrophobic context is highlighted by integral membrane metalloproteases, which cleave the peptide bonds of membrane-bound proteins (7). The reaction takes place inside the lipid bilayer by lateral diffusion (i.e., diffusion in the plane of the membrane) of the transmembrane peptide substrate into the enzyme catalytic center. Proteolysis is a hydrolytic process that requires usage of a water molecule, the quintessential hydrophilic substance. In this regard, membrane proteases are unique in that they can function in an environment that does not allow water molecules to reach the active site by simple diffusion. The recent structural investigation of several members of this enzyme class reveals the presence of a hydrophilic inner cavity that connects the catalytic center to the cytosol-exposed protein surface (Fig. 1A) (8–10). Water molecules can access this cavity, enabling the enzyme to capture its hydrophilic substrate and bring it to where it is needed for catalysis.

Fatty acid amide hydrolase (FAAH) displays another type of mechanism of action. The enzyme degrades compounds of the endocannabinoid class of lipids and terminates their neural

signaling activity (11, 12). FAAH is a functionally dimeric monotopic membrane protein with a large solvent-exposed globular body (Fig. 1B) (13). Its catalytic site is formed by a cavity that is located in the water-soluble globular region of the protein. The question then arises as to how the hydrophobic substrate is captured from the membrane and hydrolyzed by the enzyme. The three-dimensional structure of FAAH features an internal hydrophobic tunnel that connects the membrane-binding region to the active site (Fig. 1B). The endocannabinoid lipid substrate is apparently “sucked” by means of this channel, being thereby admitted into the binding site. A second channel, located on the cytoplasmic side of the protein, allows water molecules to approach the substrate, affording its hydrolysis. FAAH and intramembrane proteases exhibit two different catalytic strategies for hydrolyzing a lipophilic molecule (Fig. 1, A and B): desorption versus lateral diffusion. FAAH desorbs the substrate from the membrane and binds it inside the cytosol-exposed main body of the protein, whereas intramembrane proteases channel water molecules into the membrane-embedded active site, which can be accessed by the hydrophobic substrate through lateral diffusion.

Certain lipophile-processing hydrolytic enzymes are not membrane-associated. Therefore, unlike FAAH, they cannot use a direct desorption mechanism for recruiting their hydrophobic substrate(s). For their activity, these enzymes rely on auxiliary carrier proteins that can extract a lipophilic molecule from the membrane. Such a peculiar functionality is illustrated by saposins (14), whose crystal structures feature large and flexible hydrophobic pockets for binding different glycosphingolipids (15, 16). These proteins carry out the dual roles of recruiting the ligands from the membrane and presenting them to water-soluble hydrolytic enzymes such as arylsulfatase A or glucosylceramide- β -glucosidase in the proper orientation for efficient catalysis (14). In these enzymatic systems, the solution adopted to solve the problem of hydrolyzing a hydrophobic substrate is to uncouple the catalytic reaction from the capturing of the lipophile from the membrane, by making use of helper proteins that desorb and encapsulate the substrate and make it available to hydrolytic enzymes.

Bringing Together Bulky Substrates

What happens when the hydrophobic and hydrophilic substrates are both relatively large, bulky molecules? This situation is exemplified by the hydroxysteroid dehydrogenases (HSDs), enzymes that use NADPH (nicotinamide adenine dinucleotide phosphate, reduced) to catalyze the reduction of the keto group of glucocorticoid steroids, generating important metabolites such as cortisol, a nuclear-receptor ligand (17, 18). Members of this enzyme class are often anchored to the phospholipid bilayer through an integral N-terminal transmembrane helix (19) (Fig. 1C).

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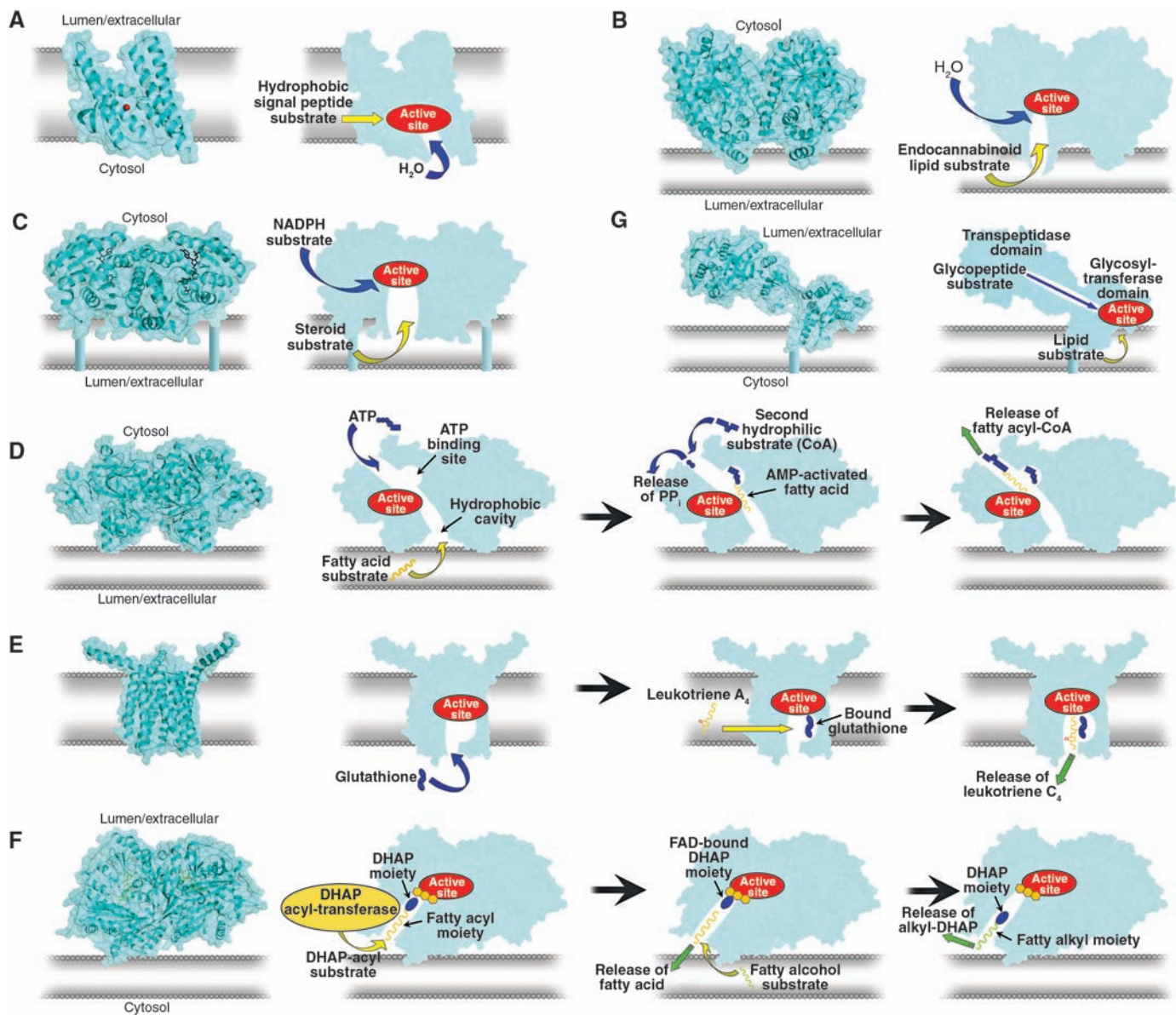


Fig. 1. Mechanisms of enzymatic reactions simultaneously involving hydrophobic and hydrophilic substrates. For each enzyme, the left panel shows the ribbon diagram and the molecular surface of the protein, whereas the right panel presents a schematic analysis of the structural basis of their mechanism of action. For the sake of simplicity, only one active site is shown for multimeric enzymes. Proteins are colored in cyan, the membrane bilayer is schematically drawn in gray, and the location of the active site is outlined in red. Transmembrane helices not visible in the crystal structures are represented by a vertical rectangle. **(A)** The catalytic center of bacterial site-2 metalloprotease is located in the bilayer and is accessible to membrane-embedded hydrophobic peptide substrates by lateral diffusion. Water, which is needed for the hydrolytic peptide cleavage, gains access through a tunnel. The red circle on the left indicates the position of the active-site Zn^{2+} atom. **(B)** The structure of dimeric membrane-anchored FAAH features a cavity open to the membrane bilayer. This cavity allows the partitioning of the lipid substrate from the membrane to the active site, which is located in the main body of the enzyme and can be readily accessed by water molecules. **(C)** The membrane association of dimeric HSD enzymes enables them to desorb the hydrophobic substrate from the membrane and reduce it by making use of a water-soluble electron donor such as NADPH (shown as black sticks in the left panel). **(D)** FACS conjugates fatty acids to coenzyme A to direct them into the cytosolic metabolism. Fatty acids (yellow) are proposed to access the catalytic cavity directly from the membrane, whereas the ATP and coenzyme A substrates (blue) reach the active site from the solvent-exposed side of the

dimeric protein. **(E)** LTC₄S illustrates another type of mechanism: the sequential binding in the same pocket of a hydrophilic substrate followed by a hydrophobic molecule. Only after glutathione (blue) binding does the catalytic cavity become suitable to bind the hydrophobic leukotriene A₄ (yellow, with reactive epoxide bond in red) substrate by lateral diffusion from the membrane phase. In the current literature, there is a contradiction regarding the orientation of LTC₄S in the membrane and, therefore, we do not specifically indicate the cytosolic and extracellular sides of the membrane. **(F)** ADPS illustrates a reaction in which a hydrophilic compound (dihydroxyacetonephosphate; DHAP, shown in blue) is encapsulated by a membrane-associated enzyme that acts on long-chain aliphatic substrates. In the first part of the catalytic cycle, this FAD-dependent enzyme (FAD is shown as a three-membered yellow ring) binds acyl-DHAP, forms a covalent bond with DHAP, and releases the acyl product (yellow) to the lipid bilayer. The reaction is completed by binding a membrane-extracted fatty alcohol (green) to generate the final alkyl-DHAP product. **(G)** The active site of peptidoglycan transglycosylase is localized at the membrane surface. This feature allows the protein to cross-link a hydrophilic glycopeptide to a membrane-embedded lipid without extracting the substrates from the aqueous and membrane phases, respectively. Another well-known and already extensively reviewed example of interfacial catalysis is provided by phospholipases that catalyze hydrolysis of phospholipids at the membrane interface (50, 51). The drawings were generated with the use of Protein Data Bank entries 3b4r (8), 1m5 (13), 1xse (20), 1v26 (25), 2uui (31), 2uuu (34), and 2olu (36).

For our analysis, we will refer to the guinea pig 11β -hydroxysteroid dehydrogenase, a representative protein of this widely studied dehydrogenase class (19, 20). Inspection of the crystal structure shows that the catalytic center is accessible both from the membrane, through a so-called steroid channel perpendicular to the membrane surface, and from the cytosol, to permit binding of the hydrophilic NADPH (20). Such architecture allows the two substrates to directly contact each other within the active site in order to facilitate the direct transfer of a hydride anion from NADPH to the steroid. The enzyme desorbs the lipophilic substrate from the lipid bilayer, catalyzes its chemical modification (a reduction) by making use of a water-soluble electron donor, and then releases the modified lipophile back to the membrane environment. A similar “desorb-and-modify” catalytic strategy underlies the reactions of several other well-known enzymes such as cholesterol oxidase (21), estrone sulphatase (22), prostaglandin synthase (23), and squalene cyclase (24).

A more elaborated mechanism of action can be identified in enzymes that catalyze the conjugation of hydrophobic and hydrophilic molecules, as in the case of bacterial fatty acyl-CoA synthetase (FACS) (4, 25, 26). This enzyme catalyzes the build-up of fatty acyl-CoA esters in the cytosol. The complicated multistep reaction of FACS shows how a catalytic site can be simultaneously accessed by three substrates coming from the cytosol and the membrane. The fatty acid is desorbed from the lipid bilayer to which the enzyme is associated and binds in a hydrophobic cavity at the interface between the two monomers of the dimeric enzyme (Fig. 1D). The elongated conformation of the fatty acid precisely positions its carboxylate group in direct contact with the binding sites for the two hydrophilic substrates, adenosine 5'-triphosphate (ATP) and coenzyme A. These two molecules bind sequentially through a hydrophilic opening exposed to the cytosolic face of the protein. The fatty acid is first activated by formation of an acyl-adenosine 5'-monophosphate intermediate and then reacts with coenzyme A to generate the final acyl-CoA product, which is eventually released to the cytosol through the hydrophilic opening. FACS exemplifies the case of an enzyme that enhances the water solubility of a lipophile by conjugation with a hydrophilic molecule (coenzyme A) in order to make it available to the cytosolic reactions.

In the above-described reactions, the common theme is the exploitation of distinct channels and binding sites for hydrophobic and hydrophilic substrates. This is not always the case, and in certain enzymes both substrates reach the same catalytic cavity in two consecutive events. The family of membrane-associated proteins in eicosanoid and glutathione metabolism represents a good example of this reaction mechanism (27–29). A prominent family member is leukotriene C_4 synthase (LTC₄S), which catalyzes the conjugation of glutathione to leukotriene A₄, an

unstable hydrophobic compound generated by arachidonic acid metabolism (30). The product of this conjugation reaction is cystenyl leukotriene or leukotriene C₄, a key mediator involved in acute and chronic inflammatory diseases of the cardiovascular and respiratory systems. The catalytic cavity of the enzyme is located at the

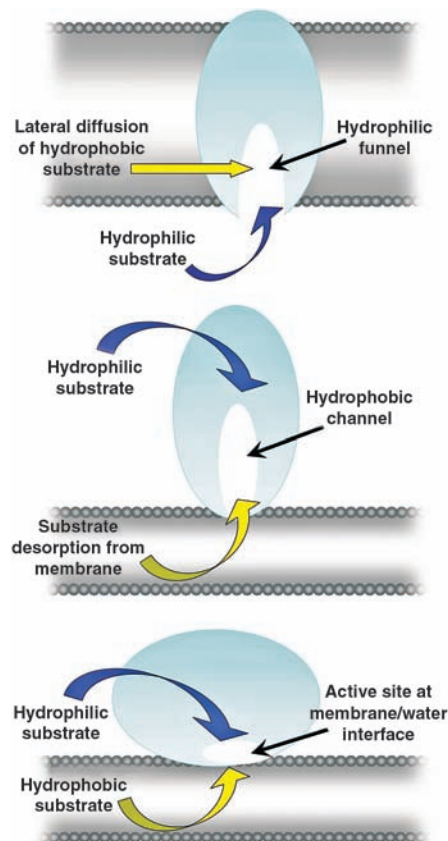


Fig. 2. Schematic view of the catalytic strategies used by enzymes working with hydrophobic and hydrophilic substrates; lateral diffusion and hydrophilic funneling (top), desorb-and-modify (middle), and working at the interface (bottom). The color coding is the same as in Fig. 1.

interface between two monomers of the biologically active LTC₄S trimer, and is buried in the lipid bilayer (Fig. 1E). As highlighted by the recently solved crystal structures, the shape and charge distribution of the active site are such that only an ordered stepwise mechanism of binding is possible (31, 32) (Fig. 1E). In the proposed catalytic scheme, glutathione first enters the binding pocket through a solvent-exposed opening, leading to a partial conformational change as well as a redistribution of the charges inside the binding site. This reorganization promotes the admission of the hydrophobic leukotriene A₄ substrate by lateral diffusion from the membrane. At this stage, the reaction can take place, generating the leukotriene C₄ product that is finally released from the enzyme. LTC₄S is unusual in that binding of the hydrophilic glutathione appears to be a prerequisite for the admission of the hydrophobic substrate. The unstable leukotriene A₄ is thus never

in danger of being exposed to water molecules that would promptly react with it. Rather, the sequential binding ensures that the substrate remains protected in its membrane environment and enters the active site only when the proper “conjugating partner” (glutathione) is already present.

Hydrophilic Intermediates and Hydrophobic Substrates

A variation on the theme is provided by alkyl-dihydroxyacetonephosphate synthase (ADPS), an enzyme involved in the biosynthesis of ether phospholipids (33). ADPS transfers the hydrophilic dihydroxyacetonephosphate (DHAP) moiety of acyl-DHAP to a highly hydrophobic long-chain fatty alcohol. From a mechanistic standpoint, the enzyme is peculiar because it uses a typical redox cofactor such as flavin adenine dinucleotide (FAD) for a nonredox reaction. ADPS is thought to be associated to the membrane surface, and its three-dimensional structure exhibits a narrow hydrophobic tunnel that runs from the membrane-protein interface to the active site (Fig. 1F) (34). The acyl-DHAP substrate, which is most likely directly donated by the preceding acyltransferase enzyme of the biosynthetic pathway, binds with its acyl chain extending into this tunnel toward the membrane surface. This binding mode promotes the reaction of its DHAP group, which forms a covalent bond with the FAD cofactor. Trapping of DHAP enables the enzyme to release the acyl product and then bind the fatty alcohol to finally generate the alkyl-DHAP compound. Thus, ADPS features the outstanding property of being able to sequester a hydrophilic intermediate (DHAP) while its lipophilic binding tunnel exchanges long-chain aliphatic ligands by direct diffusion to and from the membrane bilayer.

Working at the Membrane Surface

Intuitively, the easiest way of combining hydrophobic and hydrophilic substrates is to make them react at the membrane-water interface (Fig. 1G). A beautiful example is given by peptidoglycan transglycosylase, an enzyme involved in the biosynthesis of the bacterial cell wall (35). It is composed of two functional domains that catalyze the conjugation of a membrane-embedded lipid molecule with a polypeptide and a polysaccharide. The transpeptidase domain is responsible for the formation of a soluble glycopeptide that is then processed by the glycosyltransferase domain to generate the final product. Recent evidence from the structural analysis of this bifunctional enzyme (36) indicates that the glycosyltransferase domain is partially submerged in the lipid bilayer (Fig. 1G). Notably, its catalytic site is located exactly at the interface between the membrane and the cytosol, allowing both substrates to approach each other without being removed from their original environment. The lipid is recognized by the protein transmembrane region and placed in a productive orientation for catalysis, whereas the water-soluble glycopeptide,

deriving from the transpeptidase domain, is cross-linked to the hydrophobic substrate without entering the lipid bilayer. The final polymeric product is released, retaining its lipid moiety inside the membrane and its hydrophilic peptidoglycan head protruding from the membrane surface, fully exposed to solvent.

Challenges for the Future

The study of enzyme function remains an exciting research field because of the diverse and often unpredictable solutions that proteins develop to perform even seemingly impossible tasks. Consistently, the main concept emerging from our analysis is that there is not a unifying mechanism or working scheme that underpins the ability of enzymes to deal with a combination of hydrophobic and hydrophilic substrates. The hallmark of such diversity is the fact that these reactions can be catalyzed by integral membrane proteins, as well as by water-soluble enzymes. In this perspective, a striking observation is that there are no characteristic folding topologies that identify enzymes acting on hydrophobic-hydrophilic substrates. On the contrary, these enzymes are structurally unrelated and usually show a clear homology and evolutionary relationship with proteins that do not feature this type of enzymatic property. However, bearing in mind such a mechanistic and structural diversity, our analysis suggests a few general functional schemes illustrating how enzymes overcome the problem of acting on substrates of opposite chemical nature that typically reside within different cell compartments and environments (Fig. 2):

1) Lateral diffusion and hydrophilic funneling. When the active site is located in the lipid bilayer, the hydrophobic substrate laterally diffuses from the membrane. Hydrophilic substrates are funneled into the transmembrane portion of the enzyme across water-exposed hydrophilic channels. A fundamental feature of these membrane-embedded enzymatic systems is their topology, because the biological action exerted by the reaction product will depend on from which side of the membrane the product is released (37).

2) Desorb-and-modify. Enzymes that do not possess a membrane-embedded active site often extract their lipophilic substrates from the bilayer by direct association to the membrane surface. This process typically occurs by desorption of the lipophile by means of a tunnel or cavity that leads from the protein-membrane interface to the active site. The protein regions that mediate association to the membrane are often involved in this binding process. Alternatively, water-soluble enzymes can be assisted by carrier proteins that desorb lipophilic molecules from the membrane and make them available to the enzyme active site.

3) Working at the interface. Enzymes can have their catalytic center precisely located at the membrane surface. In this way, both hydro-

phobic and hydrophilic substrates may stay in their original environment during catalysis.

Clearly, there are several open questions that concern the function of these enzymes, especially with regard to the dynamics of both proteins and membranes and the energetics of substrate binding. Possibly, the most intriguing aspect is the desorption of a substrate from the membrane and its binding to the protein. The dynamics of this process is dictated by the association and mutual influence between the protein and the membrane phospholipids, but the biophysical bases of these phenomena remain poorly understood. New molecular dynamics methods are being developed to improve the efficacy of simulations on membrane proteins (38). Preliminary insight has been provided by simulations on saposin B (39) and on proteins that bind cholesterol [cholesterol oxidase (40) and Osh4, an oxysterol-binding protein (41)]. In these systems, the association between the membrane phospholipids and the protein is proposed to affect the conformation of a gating loop that is partially submerged into the bilayer and controls the access to the active site. The recent advances in various spectroscopic and microscopy techniques [solution and solid-state nuclear magnetic resonance (42), small-angle x-ray scattering (43), and atomic force microscopy (44)] will have a tremendous impact in this area because they will enable researchers to directly probe and visualize the conformational changes and the dynamical events underlying the processes of substrate binding at the membrane-protein interfaces.

In this context, a fascinating problem is the dependence of the substrate desorption process on the physical properties of the membranes, including their thickness and curvature, the packing of the phospholipids, and the physical state of the bilayer (i.e., whether solid-like or liquid-like). Indeed, alterations in the membrane physical properties have been shown to affect enzyme catalytic efficiency, as demonstrated for cholesterol oxidase (45). Likewise, it will be interesting to see whether the membrane-lipid composition, which differs among cell compartments, plays a role in controlling the binding affinities and catalytic efficiency, effectively acting as a modulator of these enzymatic systems. A very promising approach will be the use of nanodiscs, which provide a membrane-like environment allowing the formation of stable structures that can be investigated by different biophysical approaches (46, 47).

An in-depth analysis of substrate desorption and lateral diffusion processes must await the development of a mature model for membrane dynamics (48). Equally relevant will be the quantitative understanding of the hydrophobic effect (49) and of the energetics that favors the partitioning of a molecule from the membrane into the active site (and vice versa for product release). Investigation of the enzymatic reactions that overcome the phospholipid/water boundaries will rely on the use of emerging new techniques and on the integration of protein

chemistry with membrane biology. We suspect that these studies will lead to the development of new concepts in enzymology.

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