Kinetics and mechanisms of reactions catalyzed by immobilized lipases ☆

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Abstract *Keywords:* Enzymes; Structure/function; Reaction mechanisms; Rate expressions; Modeling

This review focuses on the kinetics of several modes of immobilization of lipases, on the mechanisms of reactions of activation of immobilized lipases, and on the kinetics and mechanisms of reactions catalyzed by immobilized lipases. A comprehensive overview of the state of the art pertaining to structural features of lipases is provided as an aid to understand immobilization, interfacial activation, and catalytic performance. General rate expressions are duly derived; more frequent simplifying assumptions are stated and the results thereof listed. Physicochemical and statistical significance of parameters in rate expressions fitted to experimental data are also discussed whenever possible.

1. Introduction

Lipases, or triacylglycerol acyl ester hydrolases (EC 3.1.1.3), are enzymes possessing an intrinsic capacity to catalyze cleavage of carboxyl ester bonds in tri-, di-, and monoacylglycerols (the major constituents of animal, plant, and microbial fats and oils). As a result of this type of reaction, carboxylic acids and alcohols with a lower number of ester bonds (and eventually glycerol) are released. In the presence of only water traces, lipases are as well able to catalyze the reverse reaction, that is, esterification. Because most fats and oils of natural occurrence are triacylglycerols of long chain (or fatty) acid residues, lipases have traditionally been termed long-chain fatty acid ester hydrolases, or alternatively esterases capable of hydrolyzing esters of oleic acid [40]. More than 50 lipases have been identified, purified and characterized to date [65], which originate in such natural sources as plants, animals, and (native or genetically engineered) micro-organisms.

The industrial versatility and unique catalytic performance of lipases have attracted increased interest throughout the world; although by 1989 they did not represent more than 4% of the global enzyme market [5], such figure has been steadily increasing ever since. Evolution encompassing protein extraction and purification methods, as well as genetic engineering and consequent cloning, is expected to further enhance this trend, thus making lipase-catalyzed processes more and more commercially feasible alternatives to bulk chemical routes. Ecological concerns have also favored more extensive applications of lipases; in fact, lipase-catalyzed reactions resemble more closely the pathways designed by nature for the metabolism of alive beings, and consequently the reaction mechanisms and processes associated therewith may be viewed as more environment-friendly than bulk chemical syntheses. Further advantages of lipases include 1) their discriminating ability (encompassing such features as stereospecificity, selectivity, and substrate specificity), which is much higher than that of inorganic catalysts and hence allows manufacture of high-

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Nomenclature

Ac	(Fatty) Acid moiety
A	Area
Al	Alcohol moiety
E_{act}	Activation energy
Es	Ester moiety
F	Acyl enzyme
I	Total number of backbone types of carboxylic acid pseudomolecules
J	Total number of backbone types of alcohol pseudomolecules
k	Lumped kinetic constant
k_1	Kinetic constant associated with adsorption of protein onto the membrane
k_{-1}	Kinetic constant associated with desorption of protein from the membrane
K_{eq}	Equilibrium constant
K_i	Inhibition constant
K_I	Acid dissociation constant
K_m	Michaëlis–Menten constant
$n_{D,i}$	Number of double bonds of the hydrocarbon moiety of the *i*-th type of glyceride
P	Protein molecule
r	Rate of hydrolysis
R	Ideal gas constant
T	Absolute temperature
V	Volume
v_{max}	Maximum rate of reaction
W	Water
*	Adsorption site on the membrane
Subscripts	
Ac	(Fatty) Acid moiety
Al	Alcohol moiety
app	Apparent
d	Deactivation
Es	Ester moiety
f	Forward direction
max	Maximum conditions
r	Reverse direction
tot	Total conditions
0	Initial (or inlet) conditions
Superscripts	
*	Normalization by the initial ester concentration
#	Indication of liquid boundary layer in the immediate vicinity of the membrane
$	Adsorption via multipoint attachment
θ	Reference conditions
Greek symbols	
α	Lumped rate parameter
β	Lumped rate parameter
ΔH	Enthalpy variation
μ	Number of carbon atoms in the fatty acid residue
Ω	Lumped parameter
Ψ	Lumped rate parameter
χ	Number of carbon atoms equivalent to each double bond in the fatty acid residue
σ	Standard deviation

added value products, and 2) their catalytic efficiency (resulting from much lower activation energies, and concomitant requirements for milder reaction conditions of temperature and pH), which reduces energy requirements and thermal damage to the reaction products.

Industrial applications of lipases have been thoroughly reviewed by Vulfson [88]. Owing to pressure towards reduction or even replacement of synthetic chemical detergents (that pose considerable environmental problems), manufacture of household detergents containing enzymes actually represents the largest industrial market for lipases because lipolytic degradation improves fatty stain removal during washing processes. Due to problems derived from compatibility with surfactants also present in detergents, extensive research was required in the near past to improve stability and activity of lipases under the alkaline conditions normally prevailing in washing processes. Protein design and genetic engineering have made it possible to produce various types of lipases in economically attractive ways, and the detergent industry has benefited from such commercial developments as Lipolase™ (NOVO Nordisk), which contains a lipase from *Humicola lanuginosa*, Lumafast™ (Genencor International), which contains a lipase from *Pseudomonas mendocina*, and Lipomax™ (Gist–Brocades), which contains a lipase from *P. alcaligenes* [40].

Most GRAS (Generally Recognized As Safe) lipases have been successfully employed in the dairy industry, mainly for acceleration of ripening in Italian-type cheeses, as well as for controlled accelerated hydrolysis of milkfat to be eventually used as additive in the manufacture of cheese-like products for flavor enhancement (e.g. dressings, soups, and sauces).

Lipases also possess a high potential to bring about controlled hydrolysis and interesterification of bulk fats and oils because of their high discriminating ability and to the purity of the products resulting therefrom. The use of such enzymes avoids the need for harsh processing conditions, such as the high temperatures required by nonenzymatic splitting and rearrangement, which lead to thermal degradation of the products (e.g. generation of off-flavors and off-colors) and, therefore, to the need for downstream refining. Lipase-based technology also allows tailoring of fats and oils for desired functional and nutritional properties, so it has accordingly been employed in large scale production of modified triglycerides and upgrading of low-value oils and fats (e.g. cocoa butter substitutes, human milkfat replacers, and nutraceutical fats enriched in polyunsaturated fatty acids). Another industrial application with a promising future is the lipase-mediated synthesis of optically pure compounds and optically active polymers (rather than racemic mixtures) for pharmaceutical and fine chemical uses. Lipase-catalyzed resolution of racemic aqueous mixtures can also occur through asymmetric hydrolysis of the corresponding esters and, in nonaqueous media, this approach can be extended to stereo- and regiospecific (trans)esterification reactions. Further industrial applications of lipases

include (but are not limited to) the production of monoglycerides to be used as emulsifiers, both in food and in personal care products (skin and sun-tan creams and bath oils), of esters of fatty acids and fatty alcohols to be used as lubricants or for oily waste treatment, and of sugar esters to be used as biosurfactants.

Although most lipases are still used in soluble form, attempts to improve the economics of the technological processes (via reutilization of the enzyme for more than a single reactor pass) and to decrease the contamination of the product (via confinement of the enzyme only to the reactor volume) have been more and more often implemented through immobilization. An extensive review on immobilized lipase reactors is available elsewhere [7]; the present review focuses specifically on the chemical reaction processes per se that take place in immobilized lipase reactors, namely immobilization of lipase, activation of lipase, and chemical reactions catalyzed by lipase.

2. Immobilization kinetics

Immobilized lipases are those lipases localized in a defined region of space, which is enclosed by an imaginary (or material) barrier that allows for physical separation of the enzyme from the bulk reaction medium and that is, at the same time, permeable to reactant and product molecules [7]. In addition to enzyme, immobilization also requires the existence of an immobilizing agent and a carrier (or barrier).

Immobilization processes can be achieved by engineering: either the *microenvironment* of the enzyme, as is the case of immobilization by attachment to a carrier (e.g. covalent attachment, hydrophobic and ion exchange adsorption and cross-linking), and immobilization by containment in a barrier (e.g. microencapsulation using lipid vesicles, containment in reversed micelles, entrapment in polymeric matrices, and confinement in ultrafiltration hollow fibers); or, alternatively, its *macroenvironment* (as modification of the reaction medium, which is achieved for example via precipitation in an organic solvent). Among all such methods, adsorption is still the most commonly used because it is the easiest to perform, and the least expensive and detrimental for activity and selectivity; adsorption basically consists of bringing an aqueous solution of lipase into intimate contact with the sorbent surface. The various immobilization protocols used with enzymes have been extensively reviewed by Kennedy and Cabral [43], and later complemented by Balcão et al. [7].

Enzyme immobilization by physical adsorption traditionally refers to binding of the enzyme via weak attractive forces to an inert carrier that was not subject to preliminary chemical derivatization. Adsorption of proteins (including enzymes) at solid/liquid interfaces is of great technical significance. The fact that lipases are activated by hydrophilic/hydrophobic interfaces, and display usually very little catalytic activity in the absence thereof, was first reported by Sarda and Desnuelle [75] and has motivated ever since a number of researchers to test polymeric membranes in attempts to find efficient carriers for lipase. Hydrophobic interactions are expected to play a major role in the adsorption of lipases onto hydrophobic membranes, not only due to the aforementioned activation but also because lipases possess an unusually high degree of hydrophobicity among enzymes when in contact with such interfaces. In particular, dehydration of hydrophobic regions of both protein and sorbent may contribute significantly to the adsorption driving force [31]; hence, preferential adsorption of lipase onto hydrophobic supports relative to other accompanying compounds in crude protein preparations (as is the case of most commercial lipase products) can lead to partial purification of lipase during adsorption [47,48,74]. Although equilibrium data relating the amount of lipase adsorbed onto the support to the amount of lipase remaining in the supernatant solution are available for *Aspergillus niger* lipases and polypropylene support [48], and *Candida rugosa* lipase and cellulose support [66], information on the kinetics associated with lipase adsorption processes is scarce. One notable exception is the work by Balcão et al. [8], who tentatively assumed that the adsorption process could be described by the following elementary (reversible) step:

$$P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P^{\#} \tag{1}$$

where P denotes a protein molecule in the bulk solution (in an essentially hydrophilic microenvironment), $P^{\#}$ a protein molecule in the liquid boundary layer at the immediate vicinity of the hydrophobic membrane (in an essentially hydrophobic microenvironment), k_1 the kinetic constant associated with adsorption of protein onto the membrane and k_{-1} the kinetic constant associated with desorption of protein from the membrane. This pseudo-adsorption process is assumed to basically consist on a reversible alteration of the protein conformation upon change of the nature of the microenvironment, with concomitant lowering of the total Gibbs' free energy of the system (as required for a spontaneous process). The forward process is expected to be largely favored when compared with the reverse process in the case of lipases owing to the unique hydrophobicity of these enzymes. On the long run, it is expected that the protein molecules in the (hydrophobic) boundary layer will interact with an increasing number of points on the carrier surface, some of which will eventually become attachment points (or true adsorption points) according to the following scheme:

$$P^{\#} + n* \overset{k}{\rightarrow} P^{\$} \tag{2}$$

where $*$ is one (of the n available) adsorption site on the membrane, $P^{\$}$ is a protein molecule adsorbed on the membrane via multipoint attachment, and k is a (lumped) kinetic

constant. (This equation encompasses the irreversibility of the adsorption process and will obviously represent an unknown number of cooperative elementary steps, one for each adsorption site.) Assumption of irreversibility of the above lumped step is in agreement with prevailing explanations that, during the adsorption process onto solid hydrophobic surfaces, the enzyme molecules, which are essentially folded in the beginning and thus able to contact the surface at a single site (or, at most, at a rather limited number of sites), begin to slowly unfold upon contact, thus allowing more of their surface to come into contact with the polymer and consequently creating a multiplicity of binding sites; although breakage of contact at a single site is likely to occur, desorption via simultaneous detachment of the protein segments from all binding sites is an extremely improbable event [47].

It has been reported that hyperactivation of certain lipases (e.g. from *Mucor javanicus*) occurs upon adsorption onto hydrophobic supports possessing large specific surface area [10,38]; this is in agreement with the postulated hypothesis that lipases recognize such well-defined hydrophobic supports as solid intefaces, and may thus become adsorbed via the external surfaces of the big hydrophobic pocket around the active center of their open and activated structure [38], without involving the active site itself. In this way, the active conformation of the lipases may be locked when physical adsorption onto the hydrophobic matrix occurs [32,68,92], thus providing a rationale for the enhancement in activity usually noticed.

The quantity of enzyme adsorbed onto a solid support depends, as expected, on the amount of enzyme directly available per unit surface of carrier during immobilization, and the activity of immobilized lipase thus increases with increasing supernatant enzyme concentration until a saturation asymptotic value is eventually reached at high enzyme concentrations. Both time and temperature play important roles in the kinetics of immobilization by adsorption, particularly with porous carriers owing to intraparticle diffusion resistance in addition to film resistance. A major disadvantage of adsorption is the leaching of protein out of the carrier during in line utilization, which can be attributed to the relative weakness of binding forces when in presence of specific components; such leaching will lead to loss of catalytic activity and to contamination of reaction products, especially at higher salt concentrations, unless the reaction mixture is hydrophobic and apolar in character.

3. Activation kinetics

Differentiation between lipases and esterases (the closest enzyme subclass) has motivated several discussions within the scientific community for over half a century. Lipases and esterases were once considered just as fat-splitting enzymes, and only the pioneering work of Sarda and Desnuelle in the fifties permitted the establishment of a functional criterion for distinction between lipases and esterases, namely their ability to be or not to be activated by oil/water interfaces, respectively. Interfacial activation of lipases is characterized by a sharp increase in lipolytic activity once the substrate solubility is exceeded [18,75], for example when the substrate starts to form an independent phase that is often dispersed as an emulsion [40]. Such qualitative hypothesis of interfacial activation of lipases was quantitatively complemented later by Desnuelle et al. [26] when the rates of lipase-catalyzed reactions observed were positively correlated with the area of available interfacial surface.

Meanwhile, many studies have been developed in attempts to understand what actually leads to, and what phenomena take place during interfacial activation of lipases. It is clear today that 1) not all lipases (and related enzymes) are activated by interfaces, as is the case of a cutinase from *Fusarium solani* ssp. *pisi* and lipases from *P. glumae* and *P. aeruginosa* [40], and 2) both the quality and the quantity of interface [71,72,86,87] play a role in the catalytic activity of these enzymes; therefore, the absence of activation of an enzyme in the presence of a given interface is not an absolute argument for conclusion that said enzyme is an esterase rather than a lipase [87]. The most widely accepted theory that has been put forward in attempts to rationalize interfacial activation of lipases asserts that a conformational change in the enzyme structure towards an activated form occurs upon contact with an oil/water interface; such dramatic increase in catalytic efficiency upon contact with natural substrates can putatively be viewed as an extension of Koshland's induced-fit theory [46,77], in that a continuum of substrate is required rather than a dispersion of solvated monomeric substrate molecules. Because fundamental explanations should focus on some level of the proteinaceous structure of lipases, comprehensive and integrated efforts have recently been made, targeted at determining the three-dimensional structures of several commercially available lipases so as to confirm, complement, and eventually generalize the original observations on pancreatic lipase by Winkler et al. [90]. Such concerted efforts were materialized in the cooperative network involving 23 European laboratories under the scope of an EC-sponsored BRIDGE T-Project, which encompassed purification, sequencing, genetic expression, cloning, crystallization, and X-ray crystallography of 11 lipases from various sources. The aforementioned R&D approaches permitted the successful resolution (at the 1 Å level) of lipases from *Rhizomucor miehei* [15,16,24], human and horse pancreas [14, 82,83], *Geotrichum candidum* [76], *C. rugosa* [35–37], *P. glumae* [62], *C. antarctica* [17], *P. mendocina* [17,80], and *Humicola lanuginosa*, *Penicillium camembertii*, and *R. delemar* [25], all in their inactive form, and some of these in their interfacially-activated form. Of the lipases scrutinized, an astonishing heterogeneity both in the catalytic properties and in the amino acid sequence was found; however, significant conservation of higher levels of protein structure

was recorded, i.e. lipases possess very low sequence homology but very high three-dimensional architectural similarity. The secondary and tertiary structures obtained by X-ray crystallography of lipases (and esterases) not exceeding 30 kDa in molecular weight were more recently confirmed by NMR of such enzymes in solution [27,65]. As discussed in detail in the following subsection, all lipases possess a serine residue at the active site. Such catalytically active Ser is, in the native structure of the enzyme, occluded by a polypeptide flap (or α-helical lid) that makes it inaccessible to substrate in aqueous media and thereby causes the enzyme to be inactive on independent, isolated substrate molecules [15,40,90].

Interfacial activation occurs when a lipase binds to a lipid interface via opening of such flap upon contact of the enzyme with an ordered interface; such opening consists in a conformational restructuring of the lipase that creates an electrophilic region (the oxyanion hole) around the aforementioned serine residue: as the helical lid rolls back from the active site, its hydrophilic side, which is exposed to the solvent in the native structure, becomes partly buried in a polar cavity previously filled by well-ordered water molecules, and simultaneously the hydrophobic side of the lid becomes completely exposed, thus greatly expanding the nonpolar surface around the active site [16]; exposure of the catalytic residues is accompanied by a marked increase in the nonpolarity of the surrounding surface, thus increasing the affinity of the three-dimensional peptide complex for the lipid substrate [40,82,83,90]. In a sense, the conformational change of the lid can be described as a simple, rigid body movement of its helical part, which consists in a translation of the center of gravity and a rotation about an axis almost parallel to that of the helix [16]. The oxyanion hole provides stabilization of the negative charge generated during the nucleophilic attack on the carbonyl bond of the substrate, and hence stabilization of the transition state intermediate during catalysis, whereas the hydrophobic side of the lid, when exposed to the lipid phase, enhances the hydrophobic interactions between the enzyme and the lipid surface [16, 40,56,83]. Structure resolution of lipases, only made possible after targeted chemical inhibition of their active site, has provided evidence that the oxyanion hole is formed by two main polypeptide chains, and that it is stabilized via van der Waals contacts formed between itself and the β_5-loop, while being accompanied by another movement in a turn following the β_4 strand leading to the correct positioning of the oxyanion hole [16,24,40,42,83,91]. The topological location of the lipase flap varies among lipases and its length and complexity usually increases with the size of the molecule. The presence and composition of the lid that covers the active site, the geometry of the catalytic triad, the structure of the oxyanion hole, and the dynamics of the lid opening have now been experimentally established as the features that impart lipases their unique structure-function characteristics, especially with respect to interfacial activation.

In attempts to model enzyme kinetics sensu latu, the Michaëlis–Menten mechanism has been recurrently used as a model; however, one of its underlying assumptions is that the enzymatic reaction must take place in an isotropic medium, i.e. both the enzyme and the substrate must be a part of the same phase. Hence, this mechanism cannot be used in its original form to model the action of lipolytic enzymes acting at the interface between a water phase and an (insoluble) lipid phase [40,67]. For this reason, a modified model has been proposed elsewhere [1,67] which consists of two steps (see Fig. 1): 1) the physical adsorption of lipase at the water lipid interface, which leads to activation of the lipase and hence to opening of the lid that would otherwise block the active site; and 2) the formation of the enzyme/substrate complex, which will eventually be hydrolyzed to give the product and regenerate the adsorbed enzyme (this second step may be described by a pseudo Michaëlis–Menten mechanism occurring on an interface, rather than in a bulk level). However, activation is not restricted to water/lipid interfaces; extensive evidence of activation of a lipase from *Mucor javanicus* upon adsorption onto such hydrophobic carriers as octyl-agarose gels [38] and polypropylene hollow-fibers [10] supports postulated hypotheses that lipases can recognize a hydrophobic/hydrophilic interface irrespective of its nature, and that exposure of the big hydrophobic pocket around the active center to substrate occurs as if a classical water/oil interface were present. Such active conformation of lipase might have been duly locked when physical adsorption onto the hydrophobic matrix did extensively occur [32,68,92], as discussed in the previous subsection.

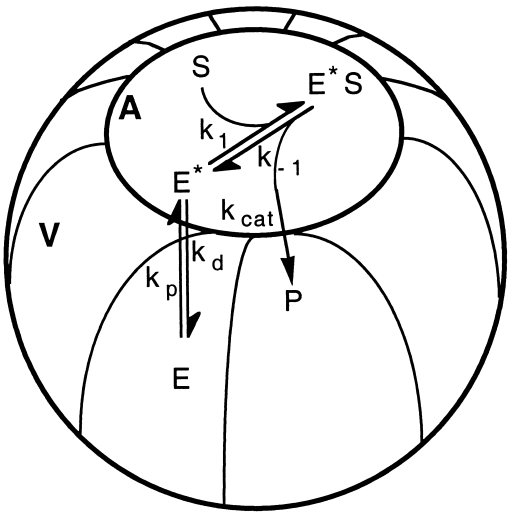

Fig. 1. Model for the description of interfacial reactions brought about by a water-soluble lipase acting on a drop of insoluble (natural) substrates. (**A**, area; **V**, volume; E, enzyme; S, substrate; E ∗ S, enzyme-substrate complex; P, product; k_1, kinetic constant associated with binding of substrate to enzyme; k_{-1}, kinetic constant associated with dissociation of substrate from the complex; k_p, kinetic constant associated with adsorption of enzyme onto the interface; k_d, kinetic constant associated with desorption of enzyme from the interface; k_{cat}, kinetic constant associated with catalysis).

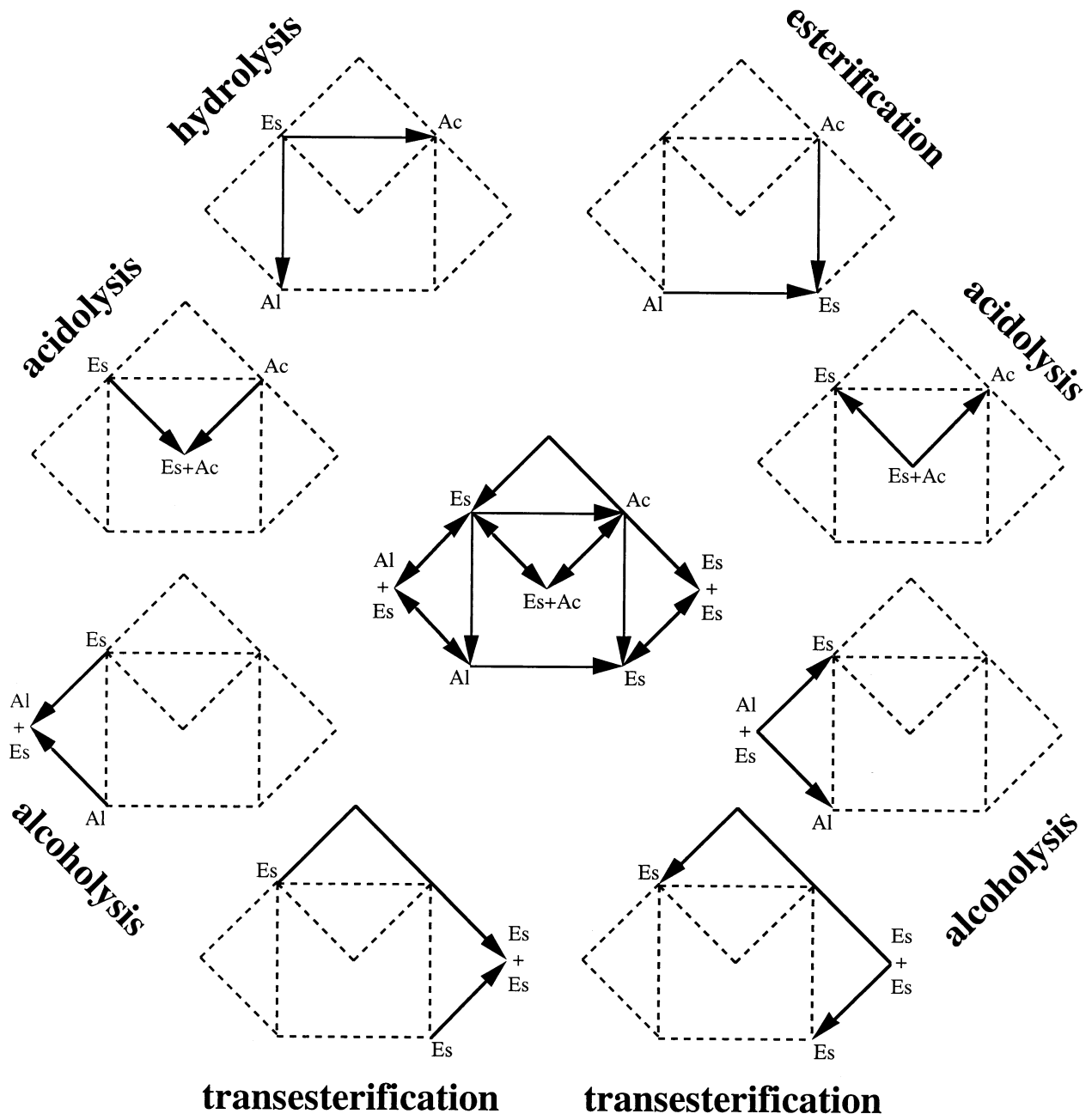

Fig. 2. Wireframe of the lipase-catalyzed reaction domain; reactants are depicted before the beginning of arrows, products are depicted after the end of arrows.

4. Reaction kinetics

4.1. Reaction types

As any other catalyst, lipases increase the rate of the reaction they catalyze with no net transformation over the time scale of the reaction and without affecting the position of chemical equilibrium. Based on the rate expression associated with a pair of reactant(s)/product(s) and catalyst, it is in principle possible to design reacting systems, and anticipate performance of existing ones, based on mechanistic (or empiric) relationships between such processing variables as time, temperature, pH and concentration of compounds present in the reaction medium.

Lipases have been originally designed by nature to catalyze cleavage of ester bonds via hydrolysis reactions (with concomitant consumption of water molecules); however, as for any chemical reaction, owing to the principle of microreversibility, the reverse reaction (i.e. ester synthesis) also takes place on the molecular level. The equilibrium conversion (but not the equilibrium constant) depends on the water content of the reaction mixture (as it does on the content of

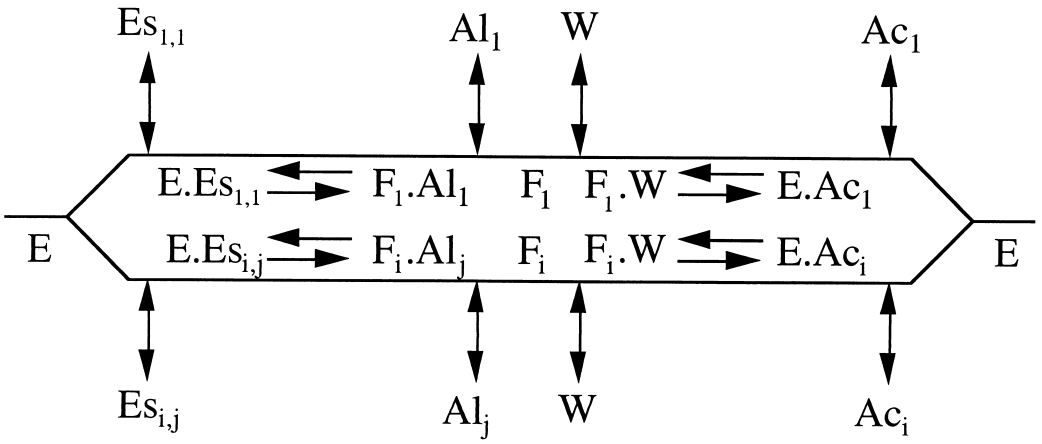

Fig. 3. Kinetic mechanism (Ping Pong Bi Bi) of lipase-catalyzed reactions involving multiple substrates/products, using Cleland's notation (E: enzyme; Es: ester moiety; Al: alcohol moiety; Ac: acid moiety; W: water; $i = 1, 2, \ldots, I$; $j = 1, 2, \ldots, J$).

any other reactant and/or product); nevertheless, since this compound is controlled easily and in a nonexpensive way, hydrolysis reactions will be promoted by macroaqueous systems and ester synthesis reactions will be promoted by microaqueous systems. These two basic processes can then be combined in a sequential fashion to give rise to a set of reactions usually termed interesterifications; depending on the particular starting point in terms of substrates, one may have acidolysis (where an acyl group is displaced between an ester moiety and a carboxylic acid moiety), alcoholysis (where an acyl group is displaced between an ester moiety and an alcohol moiety), and transesterification (where two acyl groups are exchanged between two ester moieties). In all such processes, water is needed both for maintenance of the enzyme structural integrity and for generation of the catalytic intermediate, in which case water molecules are sequentially consumed and formed, or vice-versa, with no net consumption or formation in the case of interesterification [51]. The types of reactions catalyzed by lipases are schematically depicted in Fig. 2. Although hydrolyses, esterifications and interesterifications are often tackled in an independent fashion, it should be emphasized that all such reactions are expressible as combinations of reversible hydrolyses of different reactants/products; in view of the fact that the molecule domain (i.e. the individual substrate features), rather than the reaction domain (i.e. the type of reaction actually involved in catalysis), controls the features of the reaction system, one has elected the rationale of assuming a single mechanism and hence obtaining a single, overall rate expression. This rationale is detailed below.

4.2. Intrinsic rate expressions

All 130 lipases (or related enzymes) sequenced to date [4] possess as active site a set of nonsequential Ser $\cdots$ His $\cdots$ Asp or Ser $\cdots$ His $\cdots$ Glu residues; as detailed above, this catalytic triad, a well known feature of serine proteases, is buried deeply in the interior of the enzyme structure in essentially inactive lipase molecules. The glutamate residue has been reported for the first time by Kazlauskas [42] to play a catalytic role in *G. candidum* lipase and in *Torpedo californica* acetylcholinesterase, and has meanwhile been shown to be a part of the catalytic triad in a relatively wide subfamily of lipases/esterases.

The description of the catalytic activity of enzymes in precise mathematical terms has interested many researchers throughout the years. To fully achieve this goal, an educated guess has first to be made on the elementary step reactions that occur in the system at the molecular level, i.e. a mechanism of reaction must be postulated. Such mechanism should then be translated to mathematical terms based on the statistical assumption that an elementary reaction is a result of unimolecular rearrangements or bimolecular collisions of molecules/enzymes, and that such processes are purely random and occur productively only if the free energy of the (intermediate) activated state is above a minimum threshold known as activation energy. The various concentrations will then be related on the basis of time scale considerations (viz. pseudo steady state or quasi equilibrium for the reaction intermediates), and a true macroscopic rate expression encompassing only those species that can be analytically assayed for (due to their good stability) will eventually result. The lumped parameters in such rate expression should finally be estimated via fitting of said rate expression to experimentally obtained data pertaining to the reaction progress curve. Irrespective of the type of reaction catalyzed (i.e. hydrolysis, esterification, or interesterification), the most general, accurate, and accepted description of the catalytic action of lipases is a Ping Pong Bi Bi mechanism constituted by two major steps: 1) nucleophilic attack on the substrate ester bond by the oxygen atom of the

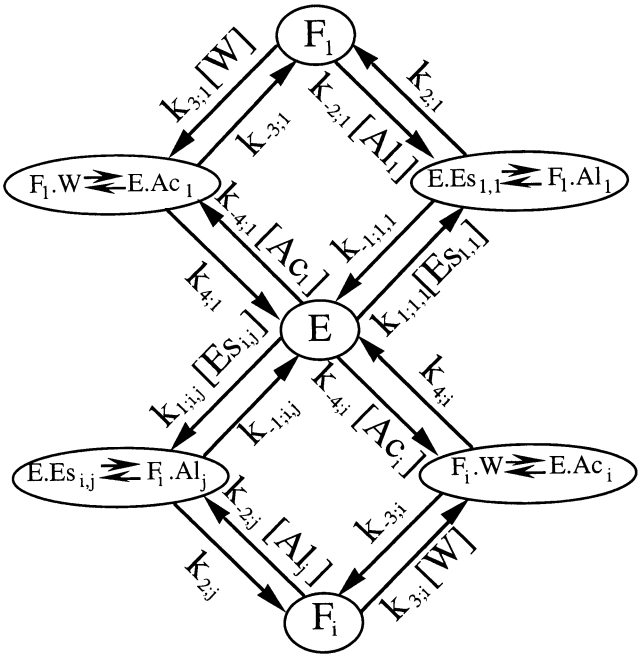

Fig. 4. King–Altman's basic figure associated with the kinetic mechanism of lipase-catalyzed reactions (E: enzyme; Es: ester moiety; Al: alcohol moiety; Ac: acid moiety; W: water; $i = 1, 2, \ldots, I$; $j = 1, 2, \ldots, J$).

hydroxyl group of Ser at the active site after opening of the lid (thus resulting in formation of an acylated enzyme complex and release of the alcohol moiety of the original substrate); and 2) hydrolysis of the acylated enzyme complex (thus resulting in formation of the product and regeneration of the enzyme). This tentative mechanism is depicted in Fig. 3 using Cleland's schematic representation.

Assume in general that a reaction catalyzed by an immobilized lipase is described by the following chemical equation:

$$Es_{i,j} + W \rightleftarrows Ac_i + Al_j \tag{3}$$

where Ac_i denotes an acid moiety of the i-th type, Al_j an alcohol moiety of the j-th type, $Es_{i,j}$ an ester moiety containing an acid residue of the i-th type and an alcohol residue of the j-th type, and W a water molecule. The basic King–Altman figure for such mechanistic process is depicted in Fig. 4. If i and j are allowed to take any integer value between 1 and I or between 1 and J, respectively, then the rate expression may be worked out from the 4^{IJ} possible $3IJ$-lined interconversion patterns after the classical King–Altman procedure [77].

Working out the fractional concentration of each enzyme form, according to the classical King–Altman's rules, and based on one of the reversible steps, say acylation/deacylation of the enzyme, then the rate of hydrolysis of any given ester moiety, $r_{m,n}$, can finally be written as:

$$r_{m,n} = \frac{\left(\begin{array}{c} (k_{1;m,n}k_{2;n}k_{3;m}k_{4;m}[Es_{m,n}][W] - \\ k_{-1;m,n}k_{-2;n}k_{-3;m}k_{-4;m}[Al_n][Ac_m]) \times \\ [E]_{\text{tot}} \prod_{j=1}^{J} \prod_{i=1}^{I} \frac{\left(\begin{array}{c} k_{-2;j}k_{-1;i,j}(k_{-3;i} + k_{4;i})[Al_j] + \\ k_{3;i}k_{4;i}(k_{-1;i,j} + k_{2;j})[W] \end{array} \right)}{\left(\begin{array}{c} k_{-2;n}k_{-1;m,n}(k_{-3;m} + k_{4;m})[Al_n] + \\ k_{3;m}k_{4;m}(k_{-1;m,n} + k_{2;n})[W] \end{array} \right)} \end{array} \right)}{\sum_{n=1}^{J} \sum_{m=1}^{I} \left(\begin{array}{c} \left(\begin{array}{c} k_{-2;n}k_{-1;m,n}(k_{-3;m} + k_{4;m})[Al_n] + \\ k_{3;m}k_{4;m}(k_{-1;m,n} + k_{2;n})[W] + \\ k_{-2;n}k_{1;m,n}(k_{-3;m} + k_{4;m})[Es_{m,n}][Al_n] + \\ k_{1;m,n}k_{3;m}k_{4;m}[Es_{m,n}][W] + \\ k_{-4;m}k_{-3;m}k_{-2;n}[Al_n][Ac_m] + \\ k_{1;m,n}k_{2;n}(k_{-3;m} + k_{4;m})[Es_{m,n}] + \\ k_{-3;m}k_{-4;m}(k_{-1;m,n} + k_{2;n})[Ac_m] + \\ k_{-4;m}k_{3;m}(k_{-1;m,n} + k_{2;n})[W][Ac_m] + \\ k_{1;m,n}k_{2;n}k_{3;m}[Es_{m,n}][W] + \\ k_{-4;m}k_{-2;n}k_{-1;m,n}[Al_n][Ac_m] \end{array} \right) \times \\ \prod_{j=1}^{J} \prod_{i=1}^{I} \frac{\left(\begin{array}{c} k_{-2;j}k_{-1;i,j}(k_{-3;i} + k_{4;i})[Al_j] + \\ k_{3;i}k_{4;i}(k_{-1;i,j} + k_{2;j})[W] \end{array} \right)}{\left(\begin{array}{c} k_{-2;n}k_{-1;m,n}(k_{-3;m} + k_{4;m})[Al_n] + \\ k_{3;m}k_{4;m}(k_{-1;m,n} + k_{2;n})[W] \end{array} \right)} \end{array} \right)} \tag{4}$$

$m = 1, 2, \ldots, I$; $n = 1, 2, \ldots, J$

where square brackets denote molar concentrations and where subscript tot refers to total conditions.

In the case of $m = n = 1$, Eq. (4) may be rewritten simply as:

$$r = \frac{v_{max,f} v_{max,r} \left([Es][W] - \dfrac{[Al][Ac]}{K_{eq}} \right)}{\left(\begin{array}{c} v_{max,r} K_{m,W} [Es] + v_{max,r} K_{m,Es} [W] + \\ \dfrac{K_{m,Ac} v_{max,f}}{K_{eq}} [Al] + \dfrac{K_{m,Al} v_{max,f}}{K_{eq}} [Ac] + \\ v_{max,r} [Es][W] + \dfrac{K_{m,Ac} v_{max,f}}{K_{eq} K_{i,Es}} [Es][Al] + \\ \dfrac{K_{m,Es} v_{max,r}}{K_{i,Ac}} [W][Ac] + \dfrac{v_{max,f}}{K_{eq}} [Al][Ac] \end{array} \right)} \tag{5}$$

where advantage was taken from Cleland's nomenclature [77] in the definition of $v_{max,f}$, $v_{max,r}$, K_{eq}, $K_{m,Ac}$, $K_{m,Es}$, $K_{i,Es}$, $K_{m,W}$, $K_{m,Al}$, and $K_{i,Ac}$. All these parameters are lumped kinetic constants, that is parameters of the rate expression that can be fitted independently (from a statistical point of view) to data available as concentrations of reactants/products. A list of rate parameters reported in the literature that use the aforementioned generic rate expression (i.e. Ping Pong Bi Bi) is given in Table 1; several characteristics of the lipases listed (e.g. source, support, and immobilization method), of the reaction medium (e.g. solvent, temperature, and pH) and of the type of reactants/

Table 1
Rate constants of immobilized lipases based on the assumption of a Ping Pong Bi Bi mechanism

Source of lipase	Support	Immobilization method	Solvent	T (°C)	pH	Es	Ac	Al	W	$v_{max,f}$	$v_{max,r}$	$K_{m,Es}$	$K_{m,W}$	$K_{m,Al}$	$K_{m,Ac}$	K_{eq}	$K_{i,Es}$	$K_{i,Ac}$	Reference
Mucor miehei	Synthetic resin	Ion exchange	*n*-Hexane	40, n.a.	n.a.	Ethyl oleate, Geranyl acetate, Isoamyl acetate	Oleic acid, Propyl acetate, Ethyl acetate	Ethanol, Geraniol, Isoamyl alcohol	Water, Propanol, Ethanol	n.a., 25.1[c]	2.125 – 342[a], 90.35[c], n.a.	76.3[b] n.a.	<0.1[b], n.a.	5.02 – 600[b]	77.18 – 450[b]	n.a., 0.95	n.a., >1.0 · 10^{16b}	40 – 1.0 · 10^{5b}	[21,22,23,70]
Candida cylindracea	n.a., FSMPM[d]	Precipitation, Adsorption	Cyclohexane, Not used	35 – 45	7.0	Trilaurin, Milkfat	Lauric acid, FFAs[e]	Dilaurin, DG[f] + MG[g]	Water	13.2 – 15811[c]	40.8 – 2650[c]	7.8[b], n.a.	n.a., 4420[b]	160[b], n.a.	17[b], n.a.	n.a., 0.209	n.a.	n.a.	[29,59]
Candida antarctica	PP[j]	n.a.	Heptane	37	n.a.	*n*-Butyl-butyrate	(R,S)-2-phenyl-propyl-butyrate	1-butanol	(R,S)-2-phenyl-1-propanol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.02	n.a.	n.a.	[58]
Pseudomonas sp.	n.a., GADERA[i]	Precipitation, Entrapment	Isooctane	35	n.a.	LABE[h]	Lauric acid	Benzyl alcohol	Water	n.a.	0.822 – 60.6[a]	n.a.	n.a.	101 – 134[b]	60 – 204[b]	n.a.	n.a.	2 – 13[b]	[34]
Pseudomonas cepacia	MPP[k], AOT-RM[m], n.a.	Precipitation + Adsorption, Containment, Precipitation	Not used, Isooctane, MIBK[p]	n.a., 25	n.a., 7.0 – 8.0	Milkfat, Olive oil, DCA[n], n.a.	Caprylic, Linoleic, Caprylic Lauric acid, 2-CAA[o]	n.a., Octanol, Decanol	n.a., Water	4.4 · 10^{-5} – 3.9 · 10^{-4} [l], 12–6240[a]	1.8 · 10^{-5} – 4.2 · 10^{-5} [l], 15000[a], n.a.	n.a., 15.8 – 27[b]	n.a.	n.a., 310[b]	n.a., 78[b]	n.a.	n.a., 350 – 636.4[b]	n.a.	[69,79,84]
Candida rugosa	PP[j]	Adsorption	Toluene	22	n.a.	n.a.	Butyric, Caproic, Caprylic, Capric, Lauric, Myristic, Palmitic, Stearic acid	Sulcatol	n.a.	n.a.	0.00796 – 0.400[a]	n.a.	n.a.	47.16 – 1181.48[b]	9.41 – 1241.2[b]	n.a.	n.a.	26.8 – 795[b]	[41]
Fusarium solani pisi	AOT-RM[m]	Containment	Isooctane	30	8.0	Hexyl acetate	Butyl acetate	Hexanol	Butanol	1056[c]	1290[c]	214[b]	n.a.	n.a.	292[b]	1.230	n.a.	n.a.	[19]

Abbreviations: n.a. ≡ not available. [a] mmol/(g · h). [b] mmol/l. [c] mmol/(l · h).
[d] FSMPM ≡ flat sheet microporous polypropylene membrane.
[e] FFAs ≡ free fatty acids. [f] DG ≡ diglycerides. [g] MG ≡ monoglycerides. [h] LABE ≡ lauric acid benzyl ester. [i] GADERA ≡ glutamic acid dioleyl ester ribitol amide.
[j] PP ≡ polypropylene. [k] MPP ≡ microporous polypropylene powder. [l] l/(h · mg). [m] AOT-RM ≡ sodium bis(2-ethylhexyl) sulphosuccinate reverse micelles.
[n] DCA ≡ decyl chloroacetate. [o] CAA ≡ chloroacetic acid. [p] MIBK ≡ methylisobutylketone.

products (e.g. Es, Ac, and Al) are also included for the sake of completeness.

4.3. Lumped rate expressions

When water is present in large excess in the reaction medium (therefore promoting hydrolysis and remaining at a virtually constant concentration), the generic Ping Pong Bi Bi rate expression depicted as Eq. (5) can be simplified to a much simpler equation that resembles the Michaëlis–Menten rate expression, namely:

$$r = \frac{v_{max,app}[Es]}{K_{m,app} + [Es]} \tag{6}$$

where $v_{max,app}$, defined as $v_{max,f}[W]/(K_{m,W} + [W])$, denotes an apparent maximum rate in the forward direction and $K_{m,\mathrm{app}}$, defined as $K_{m,Es}[W]/(K_{m,W} + [W])$, denotes an apparent Michaëlis–Menten constant. A list of rate parameters reported in the literature that use Eq. (6) as a basis of fit is given in Table 2; several pertinent characteristics of lipases (e.g. source, support, and immobilization method), of the reaction medium (e.g. solvent, temperature, and pH) and of type of reactants/products (e.g. Es, Ac, and Al) are again included. The number of reported uses of the aforementioned Michaëlis–Menten-type rate expression surpasses largely the topical uses of Eq. (5). Michaëlis–Menten kinetics has indeed played the role of a Trojan Horse with respect to the myriad of enzyme-mediated chemical processes; despite the realization that lipase-catalyzed reactions are intrinsically multisubstrate-multiproduct reactions, so kinetic models based on simplistic unisubstrate mechanisms are unlikely to provide accurate descriptions of the chemical phenomena in stake, that chemical approach is still the one of widest election.

To avoid the overparameterization problem associated with the generic Ping Pong Bi Bi mechanism when data sets of only reasonable size are available, simplifications are commonly employed following Cha's method [77]. Such method chooses as the rate-limiting step a chemical transformation involving either the enzyme or a complex thereof (see Fig. 3) and assumes that all other steps of the reaction are intrinsically much faster so that they can be considered in a state of rapid equilibrium. According to this reasoning, Eq. (5) can assume the several alternative simplified forms reported by Garcia et al. [29]; for example if the rate-controlling step is assumed to be deacylation of lipase, Eq. (5) reduces to

$$r = \frac{\dfrac{v_{max,f}}{K_{m,Es}}[Es]}{1 + \dfrac{[Es]}{K_{m,Es}} + \dfrac{[Ac]}{K_{i,Ac}}} \tag{7}$$

where the parameters $v_{max,f}$, $K_{m,Es}$, and $K_{i,Ac}$ are defined as above. If, on the other hand, acylation of lipase controls the reaction rate, the same authors have demonstrated that Eq. (5) simplifies down to a Michaëlis–Menten-type mechanism that reads

$$r = \frac{v_{max,f}[Es]}{K_{m,Es} + [Es]} \tag{8}$$

where the parameters $v_{max,f}$ and $K_{m,Es}$ were again defined previously.

Assuming that transport to/from the active site of the various species involved in the reaction occurs at essentially the same rate, or assuming that the time scales associated with transport of molecular species are very small when compared with the time scales associated with reaction (which implies that no diffusional limitations exist), stoichiometric mass balances may be written based on the chemical equation denoted as Eq. (3) that, upon combination with Eq. (4) followed by extensive algebraic rearrangement, lead to a generic rate expression of the form

$$r = \frac{\displaystyle\sum_{j=1}^{J}\sum_{i=1}^{I}\sum_{p=0}^{P}\alpha_{i,j;p}[Es_{i,j}]^p}{\displaystyle\sum_{j=1}^{J}\sum_{i=1}^{I}\sum_{q=1}^{Q}\beta_{i,j;q}[Es_{i,j}]^q} \tag{9}$$

where $P = Q$.

Fitting of rate expressions of this type usually becomes a matter of fitting ratios of polynomials; statistical decision on the degree of complexity required to fit a given data set should then be based on a model nesting procedure suggested by the various powers that appear in the polynomials, using extra sum of squares of residuals as diagnostic statistic [12]. Despite the usefulness and appropriateness of this procedure, it has received the attention of only a few researchers [9,30,49,52,53]. In the case of a single type of ester (i.e. $I = J = 1$), Eq. (9) reduces to

$$r = \frac{\alpha_0 + \alpha_1[Es] + \alpha_2[Es]^2 - \cdots}{1 + \beta_1[Es] + \beta_2[Es]^2 - \cdots} \tag{10}$$

in which case the aforementioned statistical analysis can be performed according to Bates and Watts [12].

Statistical decision on whether rate expression $r\{\alpha_0, \alpha_1, \ldots, \alpha_r; \beta_0, \beta_1, \ldots, \beta_s\}$ should be preferred to the nested rate expression $r\{\alpha_0, \alpha_1, \ldots, \alpha_{r-1}; \beta_0, \beta_1, \ldots, \beta_s\}$ then depends on the magnitude relation of the ratio of mean sum of squares to the F-statistic with *1* and *N-r-s-1* degrees of freedom at a given level of significance (say, 5% or 1%) [12]. One must not forget that the α's and β's are lumped rate parameters, that is parameters of the rate expression which can be fitted independently from a statistical point of view to data on concentration of one (or more) reactants/products; statistical independence should thus be carefully differentiated from mathematical, or functional independence.

Malcata et al. [47,49,50,52,53] have modeled their experimental data using a general Ping Pong Bi Bi mechanism

Table 2
Rate constants of immobilized lipases based on the assumption of a Michaëlis–Menten Uni Uni mechanism

Source of lipase	Support	Immobilization method	Solvent	T (°C)	pH	Es	Ac	Al	W	$v_{max,app}$	$K_{m,app}$	Reference
Mucor miehei	Synthetic resin	Ion exchange	Hexane, n.a.	30, n.a.	n.a.	Ethyl butyrate, Butyl butyrate, Isopentyl acetate, Isopentyl butyrate, Castor oil, Coconut oil	n.a.	n.a., *n*-Butanol	n.a.	0.18–10.9[a], 140–420[c]	2.4–420[b]	[60,89]
Aspergillus niger	n.a., FSMPM[e]	Precipitation, Adsorption	n.a., Not used	35–40	7.0	Butterfat	Butyric, Caprylic, Myristic, Oleic acid, FFAs[f]	n.a., Glycerol	Water	2.95–52.0[c], $4.338 \cdot 10^{-3}$ g	0.117–0.340[d], 141[b]	[28,47]
Candida rugosa	AOT-RM[h]	Containment	Isooctane	n.a. 37	7.1, n.a.	Olive oil	Oleic acid	n.a.	Water	4026[i], 33.9[c]	717[b]	[20,81]
Candida cylindracea	n.a., TFEHFM[j], APHHFM[m]	n.a., Precipitation, Entrapment	n.a., Isooctane	35–40	n.a., 7.6 – 8.0	Triolein, Tributyrin, (R,S)-CME-2-(4-IBP)PA[n], Olive oil	n.a., Oleic, Butyric acid	n.a., Glycerol	n.a., Water	n.a., 4–55[k]	9–47[b]	[32,33,57]
Vermonia galamensis seeds	PP[b]	Adsorption	Isooctane	n.a.	n.a.	Trivernolin, Triolein	Vernolic, Oleic acid	n.a.	Water	0.100–0.467[c]	58–68[b]	[2]
Rhizopus oryzae	n.a.	Precipitation	Hexane	37	7.0	Triolein	Oleic acid	n.a.	Water	4374[i]	105[b]	[63]
Pseudomonas sp.	n.a.	Precipitation	Hexane	37	7.0	Triolein	Oleic acid	n.a.	Water	2922[i]	32[b]	[63]
Mucor javanicus	n.a.	Precipitation	Hexane	37	7.0	Triolein	Oleic acid	n.a.	Water	744[i]	64[b]	[63]
Penicillium camembertii	n.a.	Precipitation	Hexane	37	7.0	Triolein	Oleic acid	n.a.	Water	132[i]	20[b]	[63]
Porcine pancreas	Lichrosorb RP-18	n.a.	Hexane, 2-Butanone	n.a.	n.a.	Tributyrin	n.a.	Hexanol	n.a.	0.48–0.54[a]	3.3–14[b]	[85]
Rhizomucor miehei	PP[l] beads	Precipitation + Adsorption	Not used	20–60	n.a.	Octyloleate	Oleic acid	Octanol	Water	735–1388[i]	600–1150[b]	[93]

Abbreviations:
n.a. ≡ not available.
[a] mmol/(h · $g_{support}$).
[b] mmol/l.
[c] μmol/min.
[d] m^2/ml.
[e] FSMPM ≡ flat sheet microporous polypropylene membrane.
[f] FFAs ≡ free fatty acids.
[g] mmol/(h · $cm^2_{support}$).
[h] AOT-RM ≡ sodium bis(2-ethylhexyl) sulfosuccinate reverse micelles.
[i] mmol/(g · h).
[j] TFEHFM ≡ tetrafluoroethylene hollow-fiber membranes.
[k] mmol/(l · h).
[l] PP ≡ polypropylene.
[m] APHHFM ≡ asymmetric (polyamide) hydrophilic hollow-fiber membranes.
[n] (R,S)-CME-2-(4-IBP)PA ≡ (R,S)-cyanomethyl ester of 2-(4-isobutylphenyl) propionic acid.

and two nested simplifications thereof (i.e. deacylation- or acylation-controlling). In the pH range 3.0 through 9.0 at 40 °C, and in the temperature range 40 through 60 °C at pH 7.0, the best fit was provided by a rate expression with the form of a Michaëlis–Menten-type mechanism with product inhibition; this rate expression actually results from the assumption of a Ping Pong Bi Bi mechanism with constant concentration of water, as derived before for Eq. (6) with deacylation as the rate-controlling step. Making use of Eq. (7) and the stoichiometric mass balances based on Eq. (3), the following simplified (lumped) rate equation can be obtained

$$r = \frac{\Omega_1[Es]}{1 + \Omega_2[Es]} \tag{11}$$

where the lumped parameter Ω_1 is defined as $v_{max,f}K_{i,Ac}/\{K_{m,Es}(K_{i,Ac} + [Es]_0)\}$ and the lumped parameter Ω_2 (which can take negative values) is defined as $(K_{i,Ac} - K_{m,Es})/\{K_{m,Es}(K_{i,Ac} + [Es]_0)\}$. The model entertained by Eq. (11) is formally equivalent to a Michaëlis–Menten rate expression for lipase-mediated hydrolysis in the presence of inhibition by free fatty acids. A list of rate parameters reported in the literature that use such lumped rate parameters is given in Table 3, coupled with several characteristics of the lipases (e.g. source, support, and immobilization method), of the reaction medium (e.g. solvent, temperature, and pH) and of the type of reactants/products (e.g. Es, Ac, and Al). Concerning Eq. (8), Malcata et al. [49] have taken advantage of the fact that $K_{m,Es} \gg [Es]$ for the proposal of a lumped form of such equation, namely:

$$r = \Psi[Es] \tag{12}$$

where the lumped parameter Ψ is defined as $v_{max,f}/K_{m,Es}$. Characteristic values of such lumped rate parameter are also given in Table 3.

As mentioned previously, when dealing with hydrolysis in the presence of excess water, its concentration is assumed to remain constant throughout the reaction timeframe; hence, the Ping Pong Bi Bi mechanism leads to a rate expression that is formally equivalent to the rate expression associated with a Michaëlis–Menten mechanism in the presence of product inhibition. Assuming deacylation of lipase as the rate-limiting step, the associated multisubstrate form of this rate expression for each kind of (fatty) acid moiety j can be written as

$$r_j = \frac{\dfrac{v_{max,j}}{K_{m,j}}[Es_j]}{1 + \displaystyle\sum_{n=1}^{M}\left\{\frac{[Es_n]}{K_{m,n}}\right\} + \sum_{n=1}^{M}\left\{\frac{[Ac_n]}{K_{i,n}}\right\}}$$

$$j = 1, 2, \ldots, M \tag{13}$$

where $[Es_j]$ and $[Ac_j]$ are the molar concentrations of ester bonds and (fatty) acids of type j, respectively, $v_{max,j}$ is the maximum rate of reaction at a saturating concentration of the ester in question, the $K_{m,j}$ are the Michaëlis–Menten constants for the esters and the K_i are the inhibition constants associated with the (fatty) acids. Derivation of Eq. (13) is based on the postulate that each type of glyceride moiety, containing a given type of fatty acid residue, competes with every other for the active site of the lipase.

From the stoichiometry of the forward reaction (hydrolysis) and in the case where the (free fatty) acid content of the inlet ester stream (as happens with regular butterfat) is negligible, Eq. (13) can be lumped [50] to yield

$$r_j^* = \frac{\Omega_{I,j}[Es_j]^*}{1 + \displaystyle\sum_{n=1}^{M}\Omega_{II,n}[Es_n]_0[Es_n]^*} \tag{14}$$

where superscript * refers to normalization by the initial ester concentration ($[Es_j]_0$); $\Omega_{I,j}$ and $\Omega_{II,j}$ are adjustable (lumped) parameters related to the values of v_{max}, K_m, and K_i of the glyceride moiety containing the j-th type of fatty acid residue, and are defined as $(v_{max,j}/K_{m,j})/\{1 + \sum_{n=1}^{M}[Es_n]_0/K_{i,n}\}$ and $(1/K_{m,j} - 1/K_{i,j})/\{1 + \sum_{n=1}^{M}[Es_n]_0/K_{i,n}\}$, respectively. As can easily be noticed from inspection of these definitions of $\Omega_{I,j}$ and $\Omega_{II,j}$, setting $M = 1$ (as would be the case if a single acid were obtained instead of a multitude of acids) leads to a definition compatible with that in Eq. (11). Lumped parameters associated with such multisubstrate rate equations are also tabulated in Table 3.

Malcata et al. [50,54,55] further considered that values for v_{max} should tentatively follow a Gaussian distribution with respect to the degree of hydrophobicity of the hydrocarbon backbone of the fatty acid residue, whereas the values of K_m and K_i should be essentially similar for all moieties. The final form of the proposed expression for lumped parameter $\Omega_{I,j}$ would then read

$$\Omega_{I,j} = \frac{\alpha}{\sigma}\exp\left\{-\frac{(n_{C,i} - \chi n_{D,i} - \mu)^2}{2\sigma^2}\right\} \tag{15}$$

where α, μ, χ, and σ are adjustable parameters and $n_{D,i}$ is the number of double bonds of the hydrocarbon moiety of the i-th type of glyceride. Parameter μ can be viewed as the number of carbon atoms in the fatty acid residue that would yield complete conversion of the native to an activated form of lipase, according to a Koshland's induced fit rationale; parameter χ is the number of carbon atoms equivalent to each double bond in the fatty acid residue; finally, parameter σ is a standard deviation.

4.4. Dependence on chemical parameters

There are several compounds that can be implicated in lipase inhibition. Laskowski and Kato [45] have listed a series of proteinaceous inhibitors of serine hydrolases (including several lipases); however, the complex interfacial activation exhibited by lipases has precluded a consensual mechanism of inhibition from being postulated. Björkling et

Table 3
Rate constants of immobilized lipases based on the assumption of a lumped Ping Pong Bi Bi mechanism

Source of lipase	Support	Immobilization method	T (°C)	pH	Es	Ac	Al	W	Ω_1 (h^{-1})	Ω_2 (M^{-1})	Ψ (h^{-1})	Ω_I (h^{-1})	Ω_{II} (M^{-1})	Reference
Aspergillus niger	PPHFM[a]	Adsorption	40 – 60	3.0 – 9.0	Butterfat	FFAs[b], Butyric, Caproic, Caprylic, Capric, Lauric, Myristic, Linoleic, Palmitic, Oleic, Stearic acid	Glycerol	Water	n.a., $0.713 \cdot 10^{-2}$ – $4.202 \cdot 10^{-2}$	n.a., $-5.055 \cdot 10^{-1}$ – $-1.490 \cdot 10^{-1}$	n.a.	n.a., $0.363 \cdot 10^{-2}$ – $5.966 \cdot 10^{-2}$	n.a., $-5.000 \cdot 10^{-1}$ – $-4.500 \cdot 10^{-1}$	[49,50,52–54]
Calf tongue	PPHFM[a]	Adsorption	40	6.0	Fractionated butterfat	FFAs[b]	Glycerol	Water	n.a.	n.a.	0.893	n.a.	n.a.	[30]
Lamb tongue	PPHFM[a]	Adsorption	40	6.0	Fractionated butterfat	FFAs[b]	Glycerol	Water	n.a.	n.a.	0.188	n.a.	n.a.	[30]
Goat tongue	PPHFM[a]	Adsorption	40	6.0	Fractionated butterfat	FFAs[b]	Glycerol	Water	n.a.	n.a.	0.215	n.a.	n.a.	[30]

Abbreviations:
n.a. ≡ not available.
[a] PPHFM ≡ Polypropylene hollow fiber membranes.
[b] FFAs ≡ free fatty acids.

al. [13] and Patkar and Björkling [64] have reported that several organophosphorous compounds inhibit lipase via irreversible phosphorylation of the serine residue at the active site. Furthermore, it is generally recognized that free fatty acids and alcohols inhibit lipase-catalyzed hydrolysis reactions [73], and it is believed that this phenomenon is due to accumulation of these compounds at the lipid/water interface thereby blocking access of triglyceride molecules to the enzyme active site.

Nonaqueous solvents can bind lipases specifically, compete with binding of substrate, or induce binding at the interface, dissociate enzyme polymers, shift equilibria between enzyme conformations, alter the amount of α-helix conformation, or react with the enzyme itself [51]. Because hydrating (or tightly bound) water is needed to maintain the structural integrity of lipases and hence their catalytic power, lipases are not active in solvents which are miscible with water (because those solvents spontaneously displace such water molecules from the proteinaceous backbones of lipases); this is the case of low molecular weight alcohols, which bring about inhibition via disruption of the three-dimensional architecture of the lipase arising from their capacity to suck bound water molecules (as hydrophobic, polar compounds). Surfactants may interfere with the catalytic function of lipases; these amphiphilic molecules can activate lipases at low concentration and inhibit them at high concentration, and a correlation has also been found between the effect of a surfactant and the extent of adsorption of lipase to the lipid interface [67].

Metal cations can have a dual performance upon lipase activity: they can either bind to free fatty acids and help remove products away from the interface (thus enhancing the activity towards hydrolysis reactions) or display an opposite effect. Since the protein surface is preferentially populated with charged and hydrophilic amino acid residues, metal ions may easily find a binding site consisting of, for example aspartic or glutamic acid. Whether or not such binding will interfere with the catalytic function of the lipase depends on the relative locations of the metal ion binding site and the active site. Presence of calcium ions usually leads to increases in hydrolysis and ester synthesis reaction rates [28,51,78]. Sodium ions have been reported to increase the activities of soluble pancreatic and *A. wentii* lipases, but to partially inhibit the lipolytic activity of two lipolytic isozymes from *A. niger* [28,51]. Reversible inhibition by heavy metal cations has been observed upon short exposures of lipases to low concentrations of these materials, whereas irreversible inhibition occurs upon long exposure to high concentration of these cations [51]. Such negative effects can be attributed to competitive binding at the active site and, when reversible, the initial conditions are often restored via addition of metal-chelating agents. Iron salts have been reported to inhibit lipases from *A. niger* [39] and *Geotrichum* sp. [44].

4.5. Dependence on physical parameters

One of the most important processing factors that affect activity of lipases is temperature. In most models of thermal deactivation, an active lipase undergoes a reversible or irreversible structural change (i.e. a temperature-induced conformational transition) to produce a catalytically inactive form [3]; because such process is essentially unimolecular, it is frequently characterized by first-order kinetics [6]. Qualitatively speaking, the decay in lipase activity upon incubation at a given temperature can be attributed to thermally-driven changes in its highly ordered three-dimensional topology: if an enzyme molecule absorbs too much energy, the tertiary structure will be disrupted and the enzyme will become denaturated, that is it will loose its catalytic activity. At high temperatures, the increase in the rate of reaction resulting from increasing the number of productive collisions between enzyme and substrate is surpassed by the increase in the rate of denaturation when temperature is increased. For this reason, a plot of the rate of reaction versus temperature usually shows a peak, often referred to as the "optimum temperature" [77,94]; however, the position of such maximum should not be interpreted in an absolute fashion because it depends on how quickly the experimenter is able to assay for the enzyme activity and which type of incubation protocol is actually selected. A more conservative concept of "optimum" temperature is the maximum temperature at which the enzyme exhibits a constant activity over a time period, at least as long as the assay time. On the other hand, substrates frequently protect an enzyme against thermal denaturation [77] and that should be considered in attempts to validate deactivation assays.

It should be noted that when inactivation is kinetically determined, rate does not actually fall with increasing temperature since it just becomes impossible to detect true initial rate because of inactivation. On the other hand, assuming that thermal denaturation is reversible and equilibrium-controlled, the classical Arrhenius (empirical) methodology for the case of temperature-dependent enzyme activities (often represented by the maximum rates of the forward reaction, $v_{max,f}$) leads to

$$\frac{v_{max,f}}{v^{\theta}_{max,f}} = \frac{\alpha e^{E_{act}/R(1/T^{\theta}-1/T)}}{1 + \psi e^{\Delta S_d/R} e^{\Delta H_d/R(1/T^{\theta}-1/T)}} \tag{16}$$

where E_{act} is the activation energy of the reaction catalyzed by the immobilized lipase, R the ideal gas constant, T the absolute temperature, α an overall (lumped) proportionality factor, ψ a lumped constant, ΔS_d the entropy of deactivation of the enzyme, and ΔH_d the enthalpy of deactivation of the enzyme, whereas superscript θ denotes reference conditions (usually the temperature at which the maximum rate is observed). Plots of the variation of activity of immobilized lipases with temperature, in a way consistent with Eq. (16), provide values for activation energy, enthalpy of deactivation and entropy of deactivation [51]. The negative slope

usually encountered when plotting Eq. (16) at high temperatures means that considerable deactivation of the enzyme takes place; such negative slope is theoretically equal to $(\Delta H_d - E_{act})/R$, where ΔH_d represents the enthalpy of deactivation of the enzyme [6]. Although individual hydrogen bonds are quite weak, typically with bond energies of 3 to 7 kcal/mol, the enthalpy of deactivation of the enzyme is quite high, ranging from 68 to 73.5 kcal/mol [6].

Another factor that strongly influences the activity of an immobilized lipase is pH. Experimental evidence [40,61] has indicated that the histidine residues at the active site must be in the proper ionic form to be able to abstract a proton from the hydroxyl group of serine (the preliminary step necessary to make a pair of electrons available for subsequent nucleophilic attack on the acyl moiety of the ester bond); an amino acid residue containing a carboxylic acid as side group (i.e. aspartic acid or glutamic acid) in the close vicinity of the catalytic site has been implicated in the formation of a three-dimensional pocket, where the substrate must bind before its ester bond can be attacked by lipase rather than directly in the reaction itself. If the aforementioned His and Asp/Glu residues are not in the proper ionic form, physical binding and subsequent chemical transformation of substrates will be prevented. It is thus expected that the effect of pH upon the catalytic stability of the lipase [6,11] will display a behavior similar to a bell-shaped curve: at very low or very high pH values, the substrate and product may either not bind the lipase or, if bound, they may not undergo chemical transformations. Such behavior is consistent with a classical Dixon–Webb rationale [6,77] with respect to enzyme activities (i.e. $v_{max,f}$) which, in the simplest form encompassing the above situation, may be written as:

$$\frac{v_{max,f}}{v^{\theta}_{max,f}} = \frac{1}{\dfrac{10^{-pH}}{K_{I,1}} + 1 + \dfrac{K_{I,2}}{10^{-\mathrm{pH}}}} \tag{17}$$

where $K_{I,1}$ and $K_{I,2}$ are acid dissociation constants, and where superscript θ denotes the maximum ever observable rate. When plotting the data reported in the literature pertaining to variation of activity of immobilized lipases with pH, the values obtained for $pK_{I,1}$ and $pK_{I,2}$ are usually closer to the pK values for dissociation of the amino acid residues Asp or Glu (3.0–5.0) and His (5.5–7.0), respectively [77]; thus, they are in agreement with the catalytic triad that constitutes the active site of lipases.

5. Final comments

Although this review has a clear focus on kinetics, it should be borne in mind that this approach can not truly be isolated from thermodynamics, even though a division line has traditionally been drawn for methodological reasons, since both kinetics and thermodynamics are in fact laid on energy considerations. According to this reasoning, all steps concerning immobilized lipase action (i.e. immobilization of enzyme, activation of enzyme, chemical transformation of reactants/products and separation of reactants/products) may be viewed as processes that proceed in the direction of decreasing overall Gibbs energy; such decrease may be either spontaneous or induced by positive contributions to the overall Gibbs energy inventory of the system arising from direct addition of work or matter. Such contributions are both enthalpic (in the form of interaction and/or kinetic components) and entropic (in the form of restriction and/or freedom components).

Immobilization of lipase may, in this regard, be spontaneous; this is the case of adsorption, where the decrease in the enthalpic components overbalance the decrease in the entropic ones. The immobilization process may alternatively be induced; this is the case of covalent attachment, entrapment, crosslinking, solvent and cold precipitation, and membrane containment. In the case of covalent attachment, entrapment, crosslinking, or solvent precipitation, the increase in Gibbs energy brought by the reactants or by the solvents provides the driving force for the decrease in Gibbs energy, that occurs a posteriori if the chemical reaction or the physical process then proceeds spontaneously. In the case of cold precipitation or physical containment, the increase in Gibbs energy is a consequence of work given to the system via volumetric work (in the form of a transmembrane osmotic pressure source) or via a hypothetical thermal engine associated therewith (in the form of heat removed to a low temperature external heat sink). The same reasoning applies to the cases of activation of lipase (where the increase in Gibbs energy results from the presence of surface energy associated with an interface, which in turn plays the role of vector for induced fit by providing an ordered array, instead of random molecular aggregates, of possible reactant molecules instead of isolated ones), with reaction (where the increase in Gibbs energy is effected by the addition of reactants), and with in situ separation (where the increase in Gibbs energy is brought about by some form of high grade energy, for example work).

When developing a single, overall rate expression, it usually contains so many parameters that it will be difficult to handle; enormous amounts of experimental data would indeed be required, and the numerical work necessary to conduct the associated nonlinear regression analysis would be prohibitive. Consequently, most kineticists have elected to either focus their attention on model systems which contain only a few different chemical species, or else analyze naturally occurring complex systems using approaches that lump the various chemical species into a few representative groups, in ways that have been duly discussed in this communication.

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