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Theoretical analysis of the activity of membrane-bound enzymes using amphiphilic or hydrophobic substrates. Application to the acyl-CoA elongases from *Allium porrum* cells and to their purification

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The theoretical approach presented in this paper shows that for membrane enzymes using amphiphilic (or hydrophobic) substrates, the value of the apparent K_M and the different shapes of the curves expressing the enzyme activities vs. the substrate concentration, depend strongly on the enzyme's environment. In addition, we provide a method allowing to determine the efficiency of the purification of such membrane-bound enzymes. By the application of the theory to the purification of the acyl-CoA elongase of leek plants, we show that the observed decrease of the activity during the purification process can not be automatically attributed to the denaturation of the proteins and similarly, when purified enzymes are incorporated into lipid vesicles, we show that the increase of the specific elongating activity is not necessarily due to the renaturation of the proteins.

Introduction

We have recently purified an acyl-CoA elongase from *Allium porrum* epidermal cells [1]. This enzyme, localized in the endomembrane system, catalyzes the synthesis of very long chain fatty acids which are the main precursors of the wax components [2]. The electrophoretic analysis of the purified elongase suggested an enrichment that was greater than 100-fold. However, the specific elongating activity was only 3-fold higher in the purified fraction than in the crude microsomes. Addition of lipid vesicles to the purified proteins allowed a 20-fold increase of the specific activity (compared to the crude microsomes). The discrepancy between the maximal 20-fold increase of the specific activity and the apparent purification factor of 100 led us to reinvestigate the various kinetic parameters obtained so far from a theoretical point of view and to reanalyse the curves expressing the activity as a function of the substrate concentration. The elongase requires two substrates, one of which is soluble in aqueous solution (malonyl-CoA), while the other is amphiphilic (stearoyl-CoA) [3]. Therefore, particular attention was paid to the concentration of long chain acyl-CoAs available for use by the enzyme rather than their 'global' concentration.

Some authors have already underlined the importance of these points. Brockman [4] measured the surface excess of palmitoyl-CoA at the air/water interface. He showed that this concentration can be written as $[S_0] \cdot [S_M]/([S_0] + K)$, where $[S_0]$ is the substrate concentration in the bulk phase, $[S_M]$ is the limiting value of the surface excess and K is an equilibrium constant. Moreover, he suggested that the velocity of the enzymes was controlled by the surface concentration of the substrate in the proximity of the enzyme, but no kinetic theory was really developped. Gatt and Bartfai [5,6] have developed theoretical studies for enzymatic reactions which utilize lipids as substrates. However, no role was assigned to the concentration of substrate in the proximity of the protein. Furthermore, the enzyme was not considered to be incorporated into a bilayer structure and, consequently, the theories developped by Gatt and Bartfai [5,6] are only applicable to very specific cases.

The theoretical approach presented in this paper allows to interpret not only the kinetic parameters of membrane-bound enzymes, but also the different shapes

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of the curves expressing the enzyme activities as a function of the overall lipophilic substrate concentration. In addition, from the theory developped herein, we provide a method allowing to determine the efficiency of the purification of membrane-bound enzymes that use amphiphilic substrates. The application of this theory to the case of the acyl-CoA elongase, is discussed in terms of the apparent denaturation of the purified enzyme in detergent micelles and its apparent renaturation when it is subsequently included in lipid vesicles.

Materials and Methods

Materials

[1-^{14}C]Stearoyl-CoA (55 Ci/mol) and [2-^{14}C]-malonyl-CoA were from Amersham. All other chemical reagents were purchased from Sigma.

Methods

Determination of membrane-bound stearoyl-CoA. 52 μg of microsomal proteins obtained as described previously [7], were incubated for 1 h at 30 °C with various concentrations of [1-^{14}C]stearoyl-CoA in a 0.08M Hepes (pH 7) buffer containing 10 mM β-mercaptoethanol, 0.5 mM NADPH, 0.5 mM NADH, 1 mM $MgCl_2$ and 2 mM DTT in a total volume of 0.1 ml.

The incubation mixtures were then centrifuged at 150000 $\times g$ for 6 min (Beckman TL100 centrifuge) and the pellets were resuspended in 3 $\times$ 50 μl of 0.08 M Hepes buffer (pH 7), containing 10 mM β-mercaptoethanol. 30 μl of this solution were used for the determination of the protein content and 75 μl were added to 425 μl of a $CHCl_3/CH_3OH$ (1 : 1, v/v) mixture. 100 μl of this homogeneous phase were used to quantify the radioactivity incorporated into the membranes.

Measurement of the acyl-CoA elongation activity. Stearoyl-CoA, used in a concentration range of 0 to 18.2 μM, was mixed with microsomes (52 μg of proteins) in a 0.08 M Hepes (pH 7) buffer containing 10 mM β-mercaptoethanol, 0.5 mM NADPH, 0.5 mM NADH, 1 mM $MgCl_2$ and 2 mM DTT in a total volume of 0.1 ml.

Another series of microsomal samples (52 μg) was incubated with the same concentrations of stearoyl-CoA in a 0.08 M Hepes (pH 7) buffer. The final volume was 0.1 ml. These mixtures were then centrifuged at 150000 $\times g$ for 5 min (Beckman TL100 centrifuge) and the pellets were resuspended in 100 μl of a 0.08 M Hepes buffer (pH 7) containing 10 mM β-mercaptoethanol, 0.5 mM NADPH, 0.5 mM NADH, 1 mM $MgCl_2$ and 2 mM DTT in a total volume of 0.1 ml. The supernatants containing the unbound stearoyl-CoA were discarded.

For both series of experiments, 5 μl of [2-^{14}C] malonyl-CoA (17 μM final concentration) were then added to each of the incubation mixtures. Incubations were carried out for one hour at 30 °C and the reactions were stopped by the addition of 0.1 ml 5 M KOH in methanol/water (1 : 9, v/v). Fatty acids were extracted and the label was determined as described previously [7].

Experiment with purified proteins. The purification procedure as well as the experimental conditions employed when the purified proteins were used, have been previously described [1].

Protein determination. Proteins were estimated by the method of Bradford [9], using BSA as the standard.

Theory

Consider the system composed of (biological) membranes surrounded by an aqueous phase, in which an amphiphilic substrate (S) is partitioned between the membrane and the aqueous phase. Let there be an enzyme (E) that uses the substrate in the aqueous phase to catalyse a reaction of the Michaelian type, characterized by the constants K_M and $V_M = k_{cat} \cdot [E]$. Let us also consider the same enzyme, incorporated into the membranes, that uses the membrane-associated substrate to catalyse the same reaction as above, by a mechanism which will be supposed to be identical and with the same constants K_M and V_M.

The problem that is posed is to know what the experimentalist will conclude when he interprets the results of $V = f([S])$ obtained in the case of the membrane-bound activity in the same manner as he would interpret those obtained for the soluble system, i.e. by expressing the appearance of a given amount of product (P_0), in a given volume (V_0), as a function of the concentration ($[S_0]$) of the substrate present in the system.

To solve this problem, let's divide the membranes into small unit volumes (V_i) in which the enzymatic reactions can take place. Each volume V_i contains S_i moles of substrate and E_i moles of enzyme.
Let

$$[E_i] = E_i/V_i = \beta_i[E_0]$$

and

$$[S_i] = S_i/V_i = f([S_0]) \cdot [S_0]$$

where E_0 and S_0 are the total numbers of moles of enzyme and substrate, respectively present in the system and $[E_0]$ and $[S_0]$ their respective global concentrations in the incubation mixture.

Since the mechanism of the reaction catalysed by the enzyme is supposed to be identical in the aqueous phase and in the membranes and to have the same constants K_M and V_M, it follows that:

$$d[P_i]/dt = k_{cat}[E_i]/(1 + K_M/[S_i])$$

where $[P_i]$ is the molar concentration of the reaction product in the volume V_i, and t is the time.

As $P_0 = \Sigma P_i$ = total number of moles of product, then we can write for the whole system:

$$d[P_0]/dt = d(\Sigma[P_i]\cdot V_i\cdot V_0^{-1})/dt$$

$$= \Sigma k_{cat}\cdot[E_i]\cdot V_i\cdot V_0^{-1}/(1 + K_M/[S_i])$$

Hence,

$$d[P_0]/dt = k_{cat}\cdot[E_0]\cdot\Sigma\beta_i V_i V_0^{-1}/(1 + K_M/f_i([S_0])\cdot[S_0]) \quad (1)$$

Eqn. 1 is a general formula which can yield different types of curves depending on the enzyme and the substrate under study.

First case

The substrate concentrations are the same in all the membrane domains:

$$f_i([S_0]) = f_j([S_0]) = \ldots = f([S_0])$$

For example, the use of the Brockman's equation [4] gives

$$f([S_0]) = [S_M]/(K + [S_0])$$

Therefore, Eqn. 1 becomes:

$$d[P_0]/dt = k_{cat}\cdot[E_0]\cdot\Sigma\beta_i\cdot V_i\cdot V_0^{-1}/(1 + K_M/f([S_0])\cdot[S_0])$$

However, the enzyme is assumed to be exclusively localized into the membrane. Therefore:

$$\Sigma\beta_i\cdot V_i\cdot V_0^{-1} = \Sigma[E_i]\cdot V_i/[E_0]\cdot V_0 = \Sigma E_i/E_0 = 1$$

Consequently, Eqn. 1 becomes:

$$d[P_0]/dt = V_M/(1 + K_M/f([S_0])\cdot[S_0]) \quad (2)$$

where $V_M = k_{cat}\cdot[E_0]$.

(a) If $f([S_0])$ is constant. If $f[S_0]$ is constant i.e. when there is a linear incorporation of the substrate into the membranes, for all $[S_0]$ we have: $[S_i]/[S_0] = a$. (For example, this situation exists for Brockman's equation [4], when the experimental conditions are such that $[S_0] \ll K$, then $a = [S_M]/K$). Under these conditions, the curve of the membrane-bound enzyme's activity vs. $[S_0]$ is hyperbolic, giving a value for V_M which is identical to that of the soluble enzyme and a value for the apparent K_M:

$$K_{M,app} = K_M/a$$

(b) If $\partial f([S_0])/\partial[S_0] < 0$. This situation is encountered for the acyl-CoA substrates, if, in Brockman's equation [4], $[S_0]$ is of the same order of magnitude as K. Here we will limit the discussion to this case. Replacing $f([S_0])$ in Eqn. 2 by its value determined using Brockman's equation ($[S_M]/K + [S_0]$), we obtain,

$$d[P_0]/dt = V_M/(1 + K_M/[S_M] + K_M\cdot K/[S_M][S_0])$$

When compared to the soluble enzyme, the curve of the membrane-bound enzyme's activity vs. $[S_0]$ is hyperbolic, giving:

$$K_{M,app} = K_M\cdot K/([S_M] + K_M) \quad \text{and } V_{M,app} = V_M/(1 + K_M/[S_M])$$

(c) If $\partial f([S_0])/\partial[S_0] > 0$. The apparent affinity (expressed by $K_M/f([S_0])$) of the enzyme for the substrate decreases when the concentration of the substrate increases. The curve of the membrane-bound enzyme's activity vs. $[S_0]$ is sigmoid. This situation would be encountered if other enzymes present in the membrane require a non negligible amount of the same substrate used by the enzyme under study (see Fig. 1).

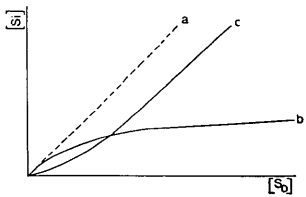

Fig. 1. Theoretical case in which $\partial f([S_0])/\partial[S_0] > 0$. Curve *a* represents a linear incorporation of the substrate into the membrane. Curve *b* represents the fixation of the substrate by enzymes other than the enzyme under study. The concentration of the remaining membrane-bound substrate available for use by the enzyme under study in these conditions, is given by curve *c*.

Second case

The variations of the substrate concentration are not the same in all the membrane domains: $f_i([S_0]) \neq f_j([S_0]) \neq f_k([S_0]) \ldots$ Here we will limit the discussion to the situation in which:

$$f_i([S_0]) = a_i, f_j([S_0]) = a_j, f_k([S_0]) = a_k \ldots$$

Under these conditions Eqn. 1 gives:

$$d[P_0]/dt = V_M\cdot\Sigma\beta_i V_i V_0^{-1}/(1 + K_M/a_i\cdot[S_0])$$

Most frequently, this equation will lead to a curve of the 'anti-cooperative' type. However, hyperbolic curves will be obtained if there is one membrane domain in which $[S_i]$ and $[E_i]$ are significantly higher than in the other domains.

Application to the determination of the purification factor

The theory presented here can be used to quantify the purification factor of an enzyme using the specific activity as reference.

For the microsomal enzyme activity, we have:

$$V_{mic} = (d[P_0]/dt)_{mic} = V_{M,mic}/(1 + K_M/f([S_0])\cdot[S_0])$$

where $V_{M,mic}$ is the maximal activity of the microsomal enzyme. The activity of the purified, delipidated enzyme can be expressed as:

$$V_{pur} = (d[P_0]/dt)_{pur} = V_{M,pur}/(1 + K_M/[S_0])$$

where $V_{M,pur}$ is the maximal activity of the purified enzyme. Therefore, assuming that the enzyme is not denatured (k_{cat} constant) during the purification process, we can calculate the purification factor Π:

$$\Pi = V_{M,pur}/V_{M,mic} = V_{pur}\cdot([S_0] + K_M)/V_{mic}\cdot([S_0] + K_M/f([S_0]))$$

By the determination of the ratio V_{pur}/V_{mic} when $[S_0] \to 0$, and knowing $f([S_0])$, it is possible to determine the purification factor since:

$$\Pi = \lim_{[S_0]\to 0} f([S_0])\cdot V_{pur}/V_{mic}$$

When $V_{mic([S_0]\to 0)}$ is not easily accessible (in the case of sigmoidal curves for example), the calculation of the purification factor requires the determination of the K_M.

In the same manner, when the purified enzyme is subsequently incorporated into a bilayer structure (phospholipid vesicles) and supposing that k_{cat} remains constant, we have:

$$V_{M,pur}/V_{M,ves} = 1 = V_{pur}\cdot([S_0] + K_M)/V_{ves}\cdot([S_0] + K_M/g([S_0]))$$

where $g([S_0])$ represents $f([S_0])$ for the bilayer structure into which the enzyme is incorporated, and V_{ves} and $V_{M,ves}$ represent the activity and the maximal activity of the purified enzyme incorporated into this bilayer structure.

Finally, the activity of the purified enzyme incorporated into this bilayer structure is given by:

$$V_{ves} = V_{pur}\cdot([S_0] + K_M)/([S_0] + K_M/g([S_0]))$$

or

$$V_{ves} = \Pi\cdot V_{mic}([S_0] + K_M/f([S_0]))/([S_0] + K_M/g([S_0]))$$

Results

Intrinsic membrane enzymes using long chain acyl-CoA as substrates are more likely to use the acyl-CoAs that are solubilized in the lipid bilayer rather than the soluble monomers and micelles [10–12]. The determination of kinetic parameters of the enzyme activity necessitates the determination of the concentration of the acyl-CoAs in the membranes. In order to estimate the global concentration of substrates in leek cell microsomes, membranes (52 μg of proteins) were incubated with increasing amounts of [1-^{14}C]stearoyl-CoA. The incubation mixture was centrifuged and the radioactivity incorporated into the pellets was determined.

Fig. 2 shows that the curve of the radioactivity associated with the membrane vs. the total concentration of labelled stearoyl-CoA in the bulk phase is linear over a large range of concentrations. Supposing the thickness of the outer leaflet to be 2.5 nm and an average diameter of microsomal vesicles of 100 nm [8] and taking into account the volume of the microsomal pellet (estimated to 1 μl), it can be estimated that the acyl-CoA concentrations in the membranes were around four hundred times higher than those in the bulk phase.

We analysed the variation of the elongating activity as a function of the concentration of the stearoyl-CoA in the aqueous phase [S_0]. This activity was measured by determining the incorporation of the [2-^{14}C]malonyl-CoA into long chain fatty acids. The results are given in Fig. 3. The curve of the activity as a function of the total stearoyl-CoA concentration is sigmoidal, which makes the estimation of K_M for the enzyme difficult. However, for stearoyl-CoA concentrations higher than 8 μM it is possible to estimate the apparent K_M. The value obtained by this method is about 6 μM (see inset of Fig. 3). The experimental data were then analysed assuming that there is a competition for the stearoyl-CoA between the elongase and other enzymes (e.g., transacylases and thioesterases which have already been

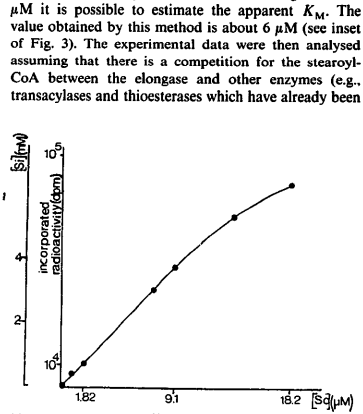

Fig. 2. Incorporation of [1-^{14}C]stearoyl-CoA into microsomes of *A. porrum* cells. 52 μg of microsomal proteins were used for each experiment. The values of the membrane-bound substrate concentration were calculated from the estimation (by excess) of the outer leaflet volume: 0.1 μl.

observed and studied in leek microsomes [13]). The theoretical curve is drawn in Fig. 3.

The theoretical curve fits with the experimental values over a large range of concentrations, but, at an acyl-CoA concentration of around 15–20 μM, the theoretical activities are higher than those actually observed. This phenomenon can be explained by the fact that the equation of the theoretical curve supposes $f([S_0])$ to be constant (linear incorporation of stearoyl-CoA into membranes; $\partial f([S_0])/\partial[S_0] = 0$), which is not the case: around 15–20 μM, $\partial f([S_0])/\partial[S_0] < 0$) and, therefore, the activity was lower than that determined theoretically.

The same experiments were carried out with microsomes preincubated with increasing amounts of stearoyl-CoA. The membranes loaded with stearoyl-CoA were sedimented and resuspended in an incubation medium containing [2-^{14}C]malonyl-CoA and all cofactors and substrates needed for the elongation, with the exception of C_{18}-CoA.

The results are shown in Fig. 3. The same sigmoid shaped curve as in the previous case was obtained. This result shows that the acyl-CoA elongase uses membrane-bound acyl-CoAs. The slight decrease of the activity observed under these conditions may be accounted for by the decrease of the amount of available stearoyl-CoA solubilized in the membranes, perhaps because of a partial redistribution of the acyl-CoA between the

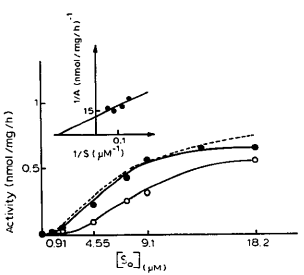

Fig. 3. Microsomal activities of elongation. ●, Elongating activities measured when 52 μg of microsomal proteins and various amounts of stearoyl-CoA were present in the incubation mixture. ○, Elongating activities measured when 52 μg of microsomal proteins preincubated with various amounts of stearoyl-CoA were used as enzyme source in an incubation mixture without stearoyl-CoA. The dashed-line represents the theoretical curve obtained using an apparent K_M of 6 μM for the stearoyl-CoA elongase and assuming a linear incorporation of the substrate into the membrane and the presence of another microsomal enzyme binding stearoyl-CoA with an equilibrium constant, K_M, of 3 μM.

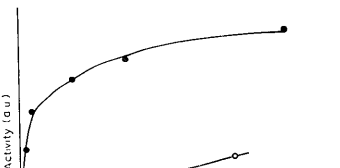

Fig. 4. Activity of the purified enzyme. ○, For each experiment, 4 μg of proteins purified as described previously (see Ref. 1) were used as the enzyme source. ●, Activity of the purified enzyme incorporated into lipid vesicles. 1.5 μg of proteins were used per essay. The lipid vesicles contain 4.6 μg of solubilized microsomal lipids and 13.2 μg of phosphatidylcholine (see Ref. 1).

resedimented microsomes and the aqueous phase containing the cofactors and the [2-^{14}C]malonyl-CoA.

From the data plotted in Fig. 2, we calculated that the concentration of the acyl-CoAs in the membrane was several hundred times higher than that in the aqueous phase. Therefore, there is a sharp increase of the actual acyl-CoA concentration in the vicinity of the membrane-bound enzyme. This may help to understand the decreased activity for a given total acyl-CoA concentration when the enzyme was solubilized and not incorporated into a bilayer structure. In other words, in the absence of a bilayer structure, the apparent K_M will be strongly increased.

Fig. 4 shows that the activity of the purified enzyme was directly proportional to the stearoyl-CoA concentration in the bulk phase in the 0 to 10 μM concentration range.

At acyl-CoA concentrations higher than 10 μM, a sharp decrease of the activity occurred and, consequently, these results could not be used to determine the K_M of the purified enzyme. However, the curve is linear for concentrations lower than 10 μM, and this property allowed to estimate the value of the K_M for the purified enzyme to be higher than 100 μM. By reconstituting a bilayer structure surrounding the purified enzyme, it was possible to significantly decrease the apparent K_M. Fig. 4 shows that under these conditions, the K_M of the enzyme is quite similar to that observed for the microsomal enzyme (1.7 μM, see Ref. 1). The slight difference in the apparent K_M of the enzyme for the polyanionic substrate may be due to the fact that, under these conditions, the protein is incorporated into vesicles

composed of neutral phospholipids (e.g. PC), and not into a natural membrane, which is usually negatively charged. Moreover, when the purified enzyme was included in lipid vesicles, i.e. when no acyl-CoA transacylases or acyl-CoA thioesterases were present with the elongase, the curve of the elongating activity as a function of the acyl-CoA concentration in the bulk phase was no longer a sigmoid as was the case for the microsomal enzyme, but was hyperbolic. Finally, it must be noted that, in good agreement with the theoretical analysis, the apparent K_M of the soluble substrate (malonyl-CoA) seems to be constant, whatever the environmental conditions of the elongase.

Discussion

The enzymology of membrane-bound enzymes has so far lagged far behind that of the soluble enzymes. In the past, most of the kinetic properties of membrane-bound enzymes have been analysed as if they were soluble and, in most cases, the theoretical treatments as well as the reasoning, have been extrapolated from what is known concerning the soluble enzymes [5,6,14].

This is a particularly unfortunate situation in the case of intrinsic membrane enzymes which use lipid substrates solubilized in the membrane. In this case, it is important to consider not only the partition coefficient, but also the concentration of membrane-bound substrate available for use by the enzyme and to define a theoretical treatment of the data which allows to account for the phenomena.

This paper shows that such an analysis is possible. We have shown that for membrane enzymes using at least one lipidic substrate, the value of the apparent K_M depends strongly on the membranous environment. Our results show that the concentration of the stearoyl-CoA in the *A. porrum* microsomal membranes may be several hundred times higher than that present in the aqueous phase and that acyl-CoA elongases use the membrane-bound stearoyl-CoA (and not that in the aqueous phase). This estimated value of the $[S_i]/[S_b]$ ratio is of course an approximation, but, whatever the true value, the important fact is that it is much greater than 100. Hence, after solubilization and purification, the enzyme was no longer included in a bilayer structure, resulting in a strong increase of the apparent K_M for stearoyl-CoA. A similar phenomenon has been already observed by Bernert [15], who described an increase in the apparent K_M of the hydroxyacyl-CoA dehydrase after the solubilization of this enzyme. His results can be explained by the theoretical analysis developped here.

The present model allows to discuss other results in the literature: we show how, knowing the K_M of the purified enzyme, it is possible to determine the partition coefficient of the substrate when this coefficient remains constant ($= a$): the curve of the apparent K_M ($= K_M/a$) vs. the amount of membrane gives a vs. the amount of the membranes under study. Recently, Fato et al. [16] have used such a method to determine the partition coefficient of ubiquinol. However, their method must be used carefully since it is applicable only when $f([S_b])$ is constant in all membrane domains and in the overall range of the substrate concentrations used.

An other interesting feature is that for a membrane enzyme with an a priori Michaelian mechanism, the shape of the curve (activity vs. substrate concentration) may be hyperbolic or sigmoid. Sigmoids have often been described in the literature for membrane enzymes which use acyl-CoA as substrate [13,17]. The explanation proposed for this phenomenon is generally that enzymes preferentially use acyl-CoAs in micelles rather than as monomers [13,17,18]. This is probably wrong since most of these enzymes must use monomers solubilized in the membrane [10–12] and not the spherical micelles remaining in the aqueous phase. We have shown that such sigmoid-shaped curves may be interpreted in terms of competition for the substrates between the enzyme under study and other proteins present in the system. We have also shown that, after purification, such an enzyme, freed of the other contaminant activities and incorporated into lipidic vesicles, could present an hyperbolic curve.

Finally, the theoretical approach may be used for defining a purification factor and, more important, it sheds some light on the question concerning the denaturation of the purified, delipidated membrane enzymes and their apparent renaturation after solubilization in a bilayer structure. Though our results cannot rule out the possibility of denaturation of the enzyme in detergent micelles, it seems that the poor activity of the purified, delipidated enzyme may simply reflect the low concentration of the substrate in the micelles. Indeed, the theoretical analysis concerning this point has been applied to the case of the acyl-CoA elongases and it gives a purification factor higher than one hundred fold, in good agreement with the electrophoretic analysis. Therefore, it seems that the catalytic capacity of the enzyme did not necessarily decrease during the purification process (see theory) and that the observed decrease of the activity can not be automatically attributed to the denaturation of the proteins, but could also be due to the sharp increase in the apparent K_M. Similarly, when purified enzymes are incorporated into lipid vesicles, the specific elongating activity could increase due to a decrease of the apparent K_M induced by a change of the enzymes environment and not necessarily because of an increase of the catalytic constant (i.e., a renaturation).

In conclusion, the model presented in this paper could be a useful tool to analyse the results obtained for membrane-bound enzymes using amphiphilic, or hydrophobic substrates.

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