Enhanced hydrolysis of phosphatidylcholine by human Group II non-pancreatic secreted phospholipase A₂ as a result of interfacial activation by specific anions

Potential role of cholesterol sulphate

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The extracellular concentration of the Group II human non-pancreatic secreted phospholipase A₂ (hnpsPLA₂) is elevated in a variety of inflammatory disorders. This enzyme is remarkable because it demonstrates almost zero activity with egg phosphatidylcholine (PC) or synthetic dioleoyl-phosphatidylcholine (DOPC) as substrate, but expresses high activity with the anionic phospholipid dioleoyl-phosphatidylglycerol (DOPG), a feature shared with the Group II enzyme from rat liver. The presence of certain membrane-bound anions can enhance hydrolysis of PC by the mammalian secreted PLA₂s. In this study the ability of various non-polar anions to stimulate DOPC hydrolysis by secreted PLA₂s has been investigated. The naturally

occurring membrane anion, cholesterol sulphate, was particularly effective in stimulating the hydrolysis of both DOPC and also 1-stearoyl-2-arachidonyl phosphatidylcholine by hnpsPLA₂. Activation of DOPC hydrolysis was also achieved with dioleoyl-phosphatidylserine (DOPS); however, DOPS was less effective than cholesterol sulphate. In contrast, the dianion dioleoyl-phosphatidic acid, a known activator of pig pancreatic PLA₂, failed to activate the human enzyme. It remains to be established whether cell plasma-membrane hydrolysis by extracellular hnpsPLA₂ can be activated *in vivo* by the presence of suitable membrane anions such as cholesterol sulphate and thus promote an inflammatory response within the cell.

INTRODUCTION

There is considerable interest in mammalian phospholipases A₂ (PLA₂s), due, in large part, to the primary role of this enzyme in the production of eicosanoids and other lipid mediators of the inflammatory response [1]. It is believed that the 85 kDa cytosolic PLA₂ plays a crucial role in the stimulus-coupled arachidonate release in cells that may be modulated by enzyme phosphorylation and changes in intracellular Ca²⁺. The 14 kDa non-pancreatic secreted enzyme is also associated with the inflammatory response. Elevated levels of this Group II enzyme were first observed in the synovial fluid of patients with rheumatoid arthritis, where it was identified as an extracellular enzyme [2]. The enzyme is also highly elevated in the serum and other extracellular fluids as a result of number of inflammatory conditions, of which the most acute example is septic shock [3].

The precise role of this 14 kDa enzyme in inflammation remains to be established; however, there are several reports that injection of this enzyme can produce such a response [4–6], but these have not been confirmed [7]. More recently the concept has been developed that such a response can be produced in activated cells as a result of plasma-membrane phosphatidylcholine (PC) hydrolysis by externally acting Group II enzyme [8,9]. It is possible that the released lysophospholipid and unsaturated fatty acid might stimulate the cytosolic 85 kDa enzyme via protein kinase C activation [10]. Although the precise mechanism by which externally added human non-pancreatic secreted PLA₂ (hnpsPLA₂) enhances eicosanoid production as a result of plasma-membrane PC hydrolysis is unclear, the basis of this

stimulated hydrolysis is of considerable interest, as it provides a possible mechanism for an excessive inflammatory response under certain conditions.

The availability of a continuous fluorescence displacement assay that allows the measurement of PLA₂ activity using normal phospholipid substrates in the absence of detergents [11] has allowed us to investigate the activity of the 14 kDa enzyme under a variety of conditions [11,12]. In particular, these studies have highlighted the barely detectable activity of the Group II enzyme from both rat liver and the recombinant human platelet with dioleoyl-phosphatidylcholine (DOPC) as substrate and in the absence of other phospholipids or detergents [13,14]. This very low activity with PC, the major type of phospholipid in the external monolayer of the plasma membrane, may be readily explained by the inability of the enzyme to bind productively to a zwitterionic interface, whereas these mammalian enzymes have a preference for anionic interfaces, particularly those involving (PG) and phosphatidylmethanol.

In the case of the Group I enzyme from pig pancreas, the enzyme shows very high affinity for phosphatidylmethanol vesicles, as compared with low-affinity binding to PC [15], and a similar preference is now indicated for the human Group II enzyme [16,17]. In contrast, the normal substrate specificity at the active site of these enzymes is minimal, with only a small difference between neutral and anionic phospholipids [16,17]. Therefore it is likely that interfacial binding and activation will play a major role in the expression *in vivo* of extracellular enzyme activity.

In this paper we have investigated the potential of a number of

Abbreviations used: FABP, fatty-acid-binding protein; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; DOPC, dioleoyl-phosphatidylcholine; SAPC, 1-stearoyl-2-arachidonyl-phosphatidylcholine; DOPE, dioleoyl-phosphatidylethanolamine; DOPG, dioleoyl-phosphatidylglycerol; DOPA, dioleoyl-phosphatidic acid; DOPS, dioleoyl-phosphatidylserine; DAUDA, 11-(dansylamino)undecanoic acid; PLA₂, phospholipase A₂; hnpsPLA₂, human non-pancreatic secreted PLA₂.

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membrane-associated non-polar anions to stimulate the ability of various 14 kDa enzymes to hydrolyse PC vesicles. The results have identified the ability of cholesterol sulphate, a naturally occurring membrane anion, to stimulate dramatically PC hydrolysis by the human Group II enzyme from essentially zero activity with pure PC. The results are discussed in terms of interfacial recognition and the ability of the Group II enzyme to hydrolyse the plasma membrane of activated cells with a possible enhancement of the inflammatory response by these cells.

EXPERIMENTAL

Materials

DOPC, dioleoyl-phosphatidylethanolamine (DOPE), 1-stearoyl-2-arachidonoyl-PC (SAPC), dioleoyl-phosphatidylglycerol (DOPG), oleic acid, arachidonic acid, cholesterol, cholesterol sulphate, cholesterol hemisuccinate, and PLA₂ from pig pancreas and *Naja naja* were obtained from Sigma. DAUDA was obtained from Molecular Probes, Eugene, OR, U.S.A. Sodium hexadecyl sulphate was obtained from Lancaster Synthesis, Morecombe, Lancs., U.K. Pure recombinant hnpPLA₂, expressed in mouse erythroleukaemic cells, was a gift from Zeneca Pharmaceuticals, Macclesfield, Cheshire, U.K. 1,2-Di[1-¹⁴C]oleoyl-phosphatidylcholine was obtained from Amersham Life Sciences.

Preparation of rat liver fatty-acid-binding protein (FABP)

The preparation of rat liver FABP from a synthetic gene expressed in *E. coli* has been described previously [18].

Preparation of rat liver PLA₂

The preparation of partially purified enzyme from rat liver was similar to that published by Aarsman et al. [19] and has been described previously [13]. After release from rat liver mitochondria by treatment with 1 M KCl and sonication, the enzyme was purified by gel filtration, followed by heparin–Sephacrose chromatography. Material eluted with high salt from heparin–Sephacrose was used for enzyme assays.

Fluorescence assay for PLA₂

The principle of this assay has been described [11]. Briefly, the assay depends on the competitive displacement of the fluorescent fatty acid probe 11-(dansylamino)undecanoic acid (DAUDA) from rat liver FABP by long-chain fatty acids, with the resulting loss of fluorescence. Bulk assay cocktails were prepared by rapid injection of 0.05 ml of phospholipid (10 mg/ml in methanol) into a buffer (9.6 ml) containing 0.1 M Tris/HCl, 0.1 M NaCl and 5 mM CaCl₂, followed by 0.1 ml of DAUDA (0.1 mM in methanol). Samples (0.975 ml) were taken for each fluorescence assay, and to this was added ~0.01 mg of FABP (0.025 ml). Optimal results were obtained if enough FABP was added to produce about a 10-fold fluorescence enhancement. When additional non-polar anions were required, they were mixed with the phospholipid in methanol before addition to assay buffer. Fluorescence assays were performed with an Hitachi F2000 fluorimeter, with excitation and emission wavelengths of 350 and 500 nm respectively. All data were recorded with a micro-computer. Calibration of displacement was performed by adding oleic acid (1 mM in methanol) to the complete assay system in the absence of enzyme.

RESULTS

Comparison of the activity of Group I and Group II enzymes on PC and PG

In the Introduction we have highlighted the importance of interfacial negative charge on the ability of the Group I PLA₂ from pancreas and the mammalian non-pancreatic Group II enzymes to hydrolyse phospholipid substrates. Using the fluorescence displacement assay, we have compared the activity of PLA₂s on PC and PG using the defined molecular species DOPC or DOPG. The results are shown in Figure 1 for a variety of PLA₂s, including three venom enzymes. It is clear that the mammalian enzymes all have a marked preference for DOPG, and this preference is due to the low activity that is expressed under these assay conditions with DOPC as substrate. This preference is most apparent in the case of human and rat Group II enzymes, and data illustrating the essentially zero rate of the rat and human Group II enzyme have been presented previously [13,14]. Figure 1 also shows rates for DOPE, as they were available for comparison, and confirm [20] the significantly lower activity expressed by the snake venom enzymes on this substrate. DOPE was not used further in the present study.

Although most of the work described in this paper uses the defined phospholipid DOPC, the lack of activity of the human Group II enzyme noted with this substrate is not due to the specific fatty acid composition of this phospholipid, because egg PC shows the same phenomenon (Figure 2). That this lack of activity with PC was not due to irreversible non-productive binding or inactivation of the Group II enzymes was demonstrated by adding DOPG to the assay after egg PC. An immediate burst of activity was observed, equivalent to that observed when DOPG alone was present (Figure 2). The same result was obtained with DOPC followed by DOPG, and the immediate response suggests that the enzyme was not bound, or was bound with low affinity, to the PC. It should be noted that it is important to use freshly prepared samples of pure PC in order to

Figure 1 Effect of phospholipid headgroup on the rate of hydrolysis by PLA₂ from various sources

The chemically defined phospholipids DOPC, DOPE and DOPG were used. For each enzyme used the rates are expressed as a percentage relative to DOPC (100%). Assays for PP, BV, NN and CA were performed at 43 °C, and the data are taken from [12]. HR and RL were assayed at 37 °C. Relative rates are given on a log scale. White bars refer to DOPC, hatched bars to DOPE and black bars to DOPG. Enzyme source abbreviations are: PP, pig pancreas PLA₂; BV, bee venom PLA₂; NN, *N. naja* PLA₂; CA, *C. atrox* PLA₂; HR, hnpPLA₂; RL, rat liver mitochondrial PLA₂. Values are means ± S.D.

Figure 2 Hydrolysis of egg PC and egg PG by hnsPLA₂

Assays were performed with hnsPLA₂ (38 ng). Trace (a) is for egg PC and no enzyme; trace (b) is for egg PG; and trace (c) is for egg PC with enzyme, followed by the addition of egg PG to give a final egg PG concentration as in (b) at 50 μ g/ml, but added at 65 s by addition of 5 μ l of 10 mg/ml egg PG in methanol.

Figure 3 Effect of cholesterol sulphate on DOPC hydrolysis by PLA₂

DOPC and cholesterol sulphate were mixed in various molar ratios in methanol before injection into the assay. Recombinant hnsPLA₂ (HR) is represented by diamonds, pancreatic PLA₂ (PP) by squares, and *N. naja* PLA₂ (NN) by triangles. Initial rates of hydrolysis were determined and are expressed in terms of specific activity. Values are means $\pm$ S.D.

observe very low activity with the Group II enzymes. Samples that were not fresh and had possibly undergone some degradation could show significant rates of hydrolysis with the hnsPLA₂ enzyme.

Effect of cholesterol sulphate on PLA₂ activity

In order to present a non-hydrolysable anion in the interface that would have a minimal effect on normal bilayer structure, we decided to investigate the effect of cholesterol sulphate. Cholesterol itself is a major lipid component of the plasma membrane, where it affects the fluidity of acyl chains. However, being a normal physiological component of the membrane, cholesterol cannot have a major deleterious effect on overall membrane structure and function, provided that the molar proportion is within normal physiological limits. Therefore cholesterol sulphate should provide a useful method of introducing negative charge, particularly since addition of cholesterol itself provides a

Figure 4 Effect of cholesterol on DOPC hydrolysis by pig pancreatic PLA₂

DOPC and cholesterol were mixed in various molar ratios in chloroform and evaporated to dryness under N₂. The lipid mixture was dissolved in methanol and injected into the assay cocktail (see the Experimental section). Initial rates of hydrolysis were determined and compared with the rate in the absence of cholesterol.

control to highlight any major effects on bilayer structure and enzyme activity.

The effect of increasing molar proportions of cholesterol sulphate to DOPC on PLA₂ activity is shown in Figure 3. Whereas only a very modest effect is seen with the pancreatic enzyme, a dramatic stimulation is seen with the human enzyme. This stimulation is maximal at a molar ratio with respect to PC of about 0.3, when up to about a 100-fold increase in rate, compared with the barely detectable control rate in the absence of cholesterol sulphate, was found. By comparison, the normal inhibitory effect of cholesterol on PC hydrolysis by the pancreatic enzyme (Figure 4) is clearly demonstrated, in which enzyme activity is decreased by an increasing molar proportion of cholesterol.

In view of the importance of the release of arachidonic acid as the first step in the biosynthesis of a variety of lipid mediators, the ability of cholesterol sulphate to stimulate SACP hydrolysis was investigated. The results (Figure 5) parallel those with DOPC and clearly demonstrate a dramatic stimulation by SACP of the human enzyme in the presence of cholesterol sulphate. Again, optimal stimulation is achieved at a molar ratio of about 0.3.

Figure 5 Effect of cholesterol sulphate on SACP hydrolysis by PLA₂

Experimental conditions, symbols and abbreviations are as for Figure 3.

Table 1 Effect of specific anions on the stimulation of DOPC hydrolysis by mammalian Group I and Group II PLA₂s

All assays were performed at a final phospholipid concentration of 50 $\mu\text{g/ml}$ at 25 °C. Rates are expressed relative to that with DOPG performed under the same conditions. The specific activity using DOPG normally varied between 100 and 500 $\mu\text{mol/min}$ per mg under these assay conditions for the enzymes using different substrate preparations. All comparisons between different PLA₂ isoforms were performed on the same substrate preparations to eliminate the possible variation of interfacial quality between different vesicle preparations. Abbreviations: CS, cholesterol sulphate; CHS, cholesterol hemisuccinate; HDS, hexadecyl sulphate; HDP, hexadecyl phosphate.

Substrate + anion (molar ratio)	PLA ₂ activity (% of rate with DOPG)		
	<i>N. naja</i>	hnpsPLA ₂	Pig pancreas
DOPG	100	100	100
DOPC	106 ± 10	< 0.1	2.1 ± 0.1
DOPC + CS (1:0.3)	110	2.2 ± 0.1	10.4 ± 0.3
DOPC + CHS (1:0.3)	110	1.1 ± 0.1	5.5 ± 0.4
DOPC + HDS (1:0.3)	106	1.7 ± 0.1	7.9 ± 0.5
DOPC + HDP (1:0.3)	—	< 0.1	1.4 ± 0.1
DOPC + DOPA (1:0.3)	110	< 0.1	6.5
DOPC + DOPS (1:0.3)	112 ± 5	1.3 ± 0.2	7.1 ± 0.4
DOPC + lyso-PG (1:0.3)	66 ± 3	1.7 ± 0.1	4.3 ± 0.6

Figure 6 Effect of cholate on DOPC hydrolysis by PLA₂

DOPC and cholate were mixed in various molar ratios in methanol before injection into assay medium. Initial rates of hydrolysis were determined. Recombinant hnpsPLA₂ (HR) is represented by diamonds, pig pancreatic PLA₂ (PP) by squares and *N. naja* PLA₂ (NN) by triangles. Values are means ± S.D.

Effect of other non-hydrolysable interfacial anions on PC hydrolysis by PLA₂

An important question was to determine the molecular specificity of the stimulation of DOPC hydrolysis by cholesterol sulphate and, in particular, how much of the stimulation was due to the presence of cholesterol as the parent molecule.

To attempt to answer these questions, two further anions were also evaluated, namely hexadecyl sulphate and cholesterol hemisuccinate. In each case, the effect of the presence of the anion at a molar ratio of 0.3 on hydrolysis rates was compared with the rate for pure DOPC only. In all experiments the concentration of phospholipid was kept constant. Table 1 shows that both hexadecyl sulphate and cholesterol hemisuccinate produced large stimulations of activity. However, the presence of a sulphate anion appears to be the more dominant stimulatory feature, making cholesterol sulphate the most effective stimulator of the

human enzyme so far discovered. The use of the dianionic hexadecyl phosphate failed to cause a stimulation of hydrolysis (Table 1).

The rate of hydrolysis of DOPC by the snake venom enzyme from *N. naja* was already high, and was not significantly stimulated by the presence of interfacial anions. This 'highly penetrating enzyme' is known to be effective against PC and also cell membranes [21,14].

Effect of dioleoyl-phosphatidic acid (DOPA), dioleoyl-phosphatidylserine (DOPS) and lysoPG on phospholipid hydrolysis

Two anionic phospholipids that are of possible importance in membrane activation to facilitate hydrolysis by the human PLA₂ are DOPA and DOPS.

DOPA may be generated by the action of phospholipase D on more complex phospholipids such as PC, or may be a precursor in phospholipid biosynthesis. Moreover, at low molar proportions with PC, it has been shown to enhance PC hydrolysis by PLA₂ from pig pancreas [16]. DOPA is a potential substrate for the fluorescence assay, which, like a pH-stat assay, will not distinguish between DOPC and DOPA hydrolysis. However, when the effect of DOPA (0.3 molar ratio) on phospholipid hydrolysis was studied, no stimulation of hydrolysis was observed with the human enzyme (Table 1), whereas, as expected [16], there was a significant stimulation of hydrolysis by the pig pancreatic enzyme. This stimulation was also observed at molar ratios of 0.05 with the pancreatic enzyme, but no stimulation of the human enzyme was observed with the same vesicle preparations (results not shown). The negative results with the human enzyme using DOPA highlights a significant qualitative difference in interfacial binding between the human and pig pancreatic enzymes, and suggests that *in vivo* phosphatidic acid may not be able to activate cell membrane hydrolysis by extracellular hnpsPLA₂.

Phosphatidylserine (PS) is already recognized as being involved in membrane activation. Thus, thrombin stimulation of platelets leads to externalization of this anionic phospholipid, where it is implicated in the blood-clotting cascade. Additionally, PS externalization may reflect cell aging and may target these cells for clearance by the macrophage system, where PS receptors exist on the cell surface [22].

When vesicles were prepared with mixtures of DOPS and DOPC at a molar ratio of 0.3, a stimulation of hydrolysis was observed (Table 1). However, DOPS was not as effective as cholesterol sulphate in stimulating fatty acid release. In order to distinguish between DOPC and DOPS hydrolysis at higher molar concentrations of DOPS, these activity measurements were also performed with 1,2-di[1-¹⁴C]oleoyl PC as substrate, and the radioactive products were identified after t.l.c. The results confirmed that the presence of DOPS caused a stimulation of DOPC hydrolysis.

In view of the very high activity observed with DOPG as substrate for hnpsPLA₂, it was decided to evaluate the effectiveness of lyso-PG in stimulating DOPC hydrolysis. At a molar ratio of 0.3, there was considerable stimulation of hydrolysis by the human enzyme, whereas stimulation of the enzyme from pig pancreas was more modest (Table 1). Lysophospholipid release is associated with anoxia and cell damage [23], and may facilitate further cellular damage by the extracellular PLA₂.

Effect of bile salts on the stimulation of PC hydrolysis by Group I and Group II enzymes

It is well established that the activity of enzymes such as pig

pancreatic PLA₂, which show low activity on zwitterionic substrates such as PC, can be dramatically stimulated in mixed vesicles containing negatively charged detergents such as cholate or SDS [24].

We have investigated the effect of the anionic detergent cholate, using our normal fluorescence assay procedure, which involves low concentrations of substrate. The effect on rate of the presence of these anions up to a molar ratio of 1.0 is shown in Figure 6, with enzymes from pig pancreas, *N. naja*, and recombinant human Group II protein. In all cases we were unable to show significant stimulation by cholate under our assay conditions. Similar lack of effects was seen with other bile acids and salts, including deoxycholate and taurodeoxycholate (results not shown).

The most obvious explanation for this lack of effect on the mammalian enzymes is the low concentrations of detergent (< 0.1 mM) used in the fluorescence assay. As a result, these particular detergents are present at concentrations well below their critical micelle concentrations, which are reported to be in the mM range. Therefore they should be present as monomers or non-micellar aggregates in the soluble phase. It was not possible to duplicate the conditions of Volwerk et al. [24] with the fluorescence assay, because of the high concentrations of lipid and detergent that were used. The bile-acid/salt experiments provide a useful control in that they highlight the importance of appropriate non-polar anions being incorporated into the vesicle interface, rather than being in solution, before they can have an effect on enzyme activation.

DISCUSSION

A comparison of the activity of Group I and Group II PLA₂s reveals major differences in activity on PC and PG (Figure 1). Since for the pancreatic and human Group II enzymes it has been shown that differences in substrate preferences during hydrolysis (k_{cat}/K_m) are small [16,17], the expressed activity under conditions using the fluorescence assay must to a large extent reflect interfacial binding and activation. It is interesting that the ratios of expressed activity on DOPG:DOPC for the enzymes from human, pig pancreas, *N. naja* and *Crotalus atrox* were ~ 10³, 50, 1 and 0.2 respectively under these assay conditions, and this correlates with the pI values of these proteins of > 10.5 and 7.4 for the two mammalian enzymes, whereas the venom enzymes have pI values of about 5 [25,26]. Clearly the charge distribution on the protein, and especially the nature of the charge on the interfacial binding surface, plays an important role in apparent substrate preference. This cationic charge distribution has been previously highlighted for the human enzyme [27] and analysed in more detail in terms of electrostatic forces for several isoforms of PLA₂ [26].

The almost zero rate of hydrolysis of pure PC observed with the extracellular hnpPLA₂ is of considerable interest, as this phospholipid is the major phospholipid in the outer monolayer of the plasma membrane and also in plasma lipoproteins. This lack of activity would mean that such membranes and lipoproteins should normally be refractory to hydrolysis by this extracellular enzyme, and we have previously demonstrated that, unlike the *N. naja* venom enzyme, the human enzyme is unable to hydrolyse the cell membrane of intact hepatocytes [14].

Although there is considerable circumstantial evidence linking elevated extracellular levels of Group II PLA₂ to tissue inflammation, there is little definitive evidence to suggest a direct cause-and-effect relationship between the extracellular activity of this enzyme and an inflammatory response in tissues. It would appear that the increased release of the secreted enzyme as a result of an

inflammatory change may more reflect a degradative role of this enzyme in the removal of foreign organisms and damaged tissue [28]. Indeed, it has been proposed that the liver is a major source of enzyme [29] and that this PLA₂ may be acting as an acute-phase protein [30]. In this context of cell degradation, the very marked apparent preference of this enzyme for negatively charged phospholipids, especially PG, as a reflection of interfacial binding may be significant, as such negatively charged phospholipids are exposed on cell damage. In addition, this enzyme is activated by BPI (bactericidal/permeability-increasing protein) to allow bacterial phospholipid degradation [31].

An important question is whether under certain circumstances this extracellular human enzyme is able to hydrolyse intact cell membranes. In this paper we have investigated the possibility of physiological non-polar anions activating PC hydrolysis, and we have highlighted, in particular, the potential for activation by cholesterol sulphate. Although cholesterol sulphate has not yet been directly implicated in this type of membrane activation, it illustrates the principle of enhanced PC hydrolysis by an anionic lipid that cannot be a substrate but must promote interfacial binding. Moreover, cholesterol sulphate has previously been implicated in the process of sperm capacitation, where it can account for as much as 20 % of the sperm head surface area and have a role in membrane fusion [32]. It is noteworthy that a recent report states that cholesterol sulphate is able to activate one isoform of protein kinase C, being more effective than PS, whereas cholesterol was without effect [33].

It is possible that the mammalian Group II enzyme may show specificity for the sulphate ion. This Group II enzyme is known to bind to heparin and heparan sulphate proteoglycan at a site remote from the active site [34,35], and attachment to a heparan sulphate on the cell surface appears to be important in membrane hydrolysis and the production of inflammatory lipids [36]. Inspection of the amino acid sequence for the human enzyme reveals a potential heparin-binding site at positions 53–58 (KRLEKR).

A comprehensive study of the molecular basis of interfacial binding by the human enzyme may allow a greater understanding of the possible role of this enzyme in inflammation and inflammatory disorders. The availability of a synthetic gene for this enzyme expressed in *Escherichia coli* [37] will allow the necessary site-directed-mutagenesis approach to investigate this problem.

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