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Accelerated Evolution and Molecular Surface of Venom Phospholipase A_2 Enzymes

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Abstract. Multiple phospholipase A_2 (PLA_2) isoenzymes found in a single snake venom induce a variety of pharmacological effects. These multiple forms are formed by gene duplication and accelerated evolution of exons. We examined the amino acid sequences of 127 snake venom PLA_2 enzymes and their homologues to study in which location most natural substitutions occur. Our data show that hot spots of amino acid substitutions in this group of proteins occur mostly on the surface. A logistic model correlating the substitution rates of each amino acid residue with their surface accessibility indicates that the probability of natural substitutions occurring in the fully exposed residue is 2.6–3.5 times greater than that of substitutions occurring in buried residues. These surface substitutions play a significant role in the evolution of new PLA_2 isoenzymes by altering the specificity of targeting to various tissues or cells, resulting in distinct pharmacological effects. Thus natural substitutions in PLA_2 enzymes, in contrast to popular belief, are not random substitutions but appear to be directed toward modifying the molecular surface.

Key words: Protein evolution — Snake venom — Surface accessibility — Natural substitutions — Toxin — Pharmacological effects

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Introduction

Snake venom phospholipase A_2 (PLA_2) enzymes are esterolytic enzymes which hydrolyze glycerophospholipids at the *sn*-2 position of the glycerol backbone, releasing lysophospholipids and fatty acids. So far, several hundred snake venom enzymes have been purified and characterized. They share similarity in structure and catalytic function with mammalian enzymes. However, in contrast to mammalian enzymes, many are toxic and induce a wide spectrum of pharmacological effects (Harris 1985; Rosenberg 1990; Kini 1997). A single snake venom contains multiple forms of PLA_2 enzymes (Braganca and Sambray 1967; Salach et al. 1971; Vishwanath et al. 1987; Takasaki et al. 1990a). These isoenzymes exhibit distinctly different pharmacological effects. An examination of PLA_2 genes indicates that mutations occur in exons more frequently than in introns (Ogawa et al. 1992; Nakashima et al. 1993, 1995; Nobuhisa et al. 1996). Thus it is proposed that the multiple forms of PLA_2 enzymes and their pharmacological capabilities have evolved through gene duplication and accelerated evolution.

One of the well-accepted doctrines of our time is that mutation in nature is a random process. These mutations are not truly distributed at random; some gene loci mutate faster than others do and there are hot spots where some residues are substituted more often than others. However, the role of hot spots in evolution of proteins or organisms is not yet clear. We examined the natural sub-

stitutions in snake venom PLA_2 enzymes and the relationship between the frequency of substitution and the surface accessibility of amino acid residues.

Methods

Protein Sequences

The complete sequences of PLA_2 enzymes and their homologues are given elsewhere (Danse et al. 1997). Consensus sequences for class I and class II PLA_2 enzymes were determined based on the most common amino acid residue present in a particular location after optimum sequence alignments. The consensus sequences thus obtained are as follows [The positions indicated by an asterisk show deletions in most proteins; 51.6 ± 20.1 ($n = 5$) of 68 and 55.3 ± 2.5 ($n = 3$) of 58 proteins of class I and class II, respectively. These position pairings were ignored in the logistic model.]: class I [subgroups 1A and 1A′ (see Danse et al. 1997)], NLYQFKNMIQCANPGSRPWWHYADYGCYC-GRGGSGTPVDELDRCCQVHDDCYGEAEKK*GCYPKLTLYSYE-*C*SQGTPT*C*NGKTKCQRFVCDCDRAAAKCFAKAPYNDAN-YNIDTKKRCQ; and class II [subgroups IIA and IIA′ (see Danse et al. 1997)], SLLQFGKMILKETGKSGIWSYSSYGCYCGWGGQGKPK-DATDRCCFVHDCCYGKVTGCNPKLDRYSYSWENGDIVCGG*-DNPCLKEICECDRAAAICFRDNLDTYDKK*YWFYPDSNC*KEE-SEPC. Percentage substitution at each position was calculated based on the frequency of replacement of each residue compared with consensus sequences for each class of PLA_2 enzymes.

Surface Accessibility

Surface accessibility of amino acid residues (Connolly 1981) was determined using Insight II software on a Silicon graphics workstation. Since individual amino acid residues are of different sizes, we calculated the relative accessibility of each residue as follows:

$$\text{Relative surface accessibility} = \frac{\text{Surface accessibility in the native structure}}{\text{Surface accessibility in the tripeptide sequence}} \times 100$$

Surface accessibility of an amino acid in a tripeptide (Ala–Xaa–Ala) in an extended conformation was determined using the same software.

Logistic Regression Model

The following logistic regression model was used to study the relationship between the probability of substitution of a given residue and its relative surface accessibility. Let Y takes value 1 if a substitution occurs in an amino acid residue and 0 if no substitution occurs. Let a be the number of substitutions in a particular position and b be the total number of sequences in the class of PLA_2 enzymes. Then for a given relative surface accessibility, a of the Y values are 1 and $(b - a)$ of the Y values are 0. Let θ be the probability that an amino acid residue is substituted. Now we represent the dependence of the probabilities for this dichotomy variable, Y, on the relative surface accessibility, x.

Let $L = \log[\theta/(1 - \theta)]$, where log is the natural logarithmic function. Since L takes the logarithm of the odd ratio, $\theta/(1 - \theta)$, it is sometimes referred to as the logit of θ. A simple way to represent the dependence of the logit of θ on x is through a linear model as follows.

$$L = \log[(\theta/(1 - \theta)] = \beta_0 + \beta_1 x \quad (1)$$

where β_0 and β_1 are unknown parameters. Rewriting Eq. (1), we see that the postulated model for dependence takes the form

$$\theta = \exp(\beta_0 + \beta_1 x)/[1 + \exp(\beta_0 + \beta_1 x)] \quad (2)$$

Therefore we can get an estimate for the probability of an amino acid residue being substituted if we can obtain estimates for β_0 and β_1.

Consider the ith observation Y_i. The conditional distribution of Y_i given x_i follows a binomial distribution with size 1 and probability given by the conditional mean θ_i, where $\theta_i = \exp(\beta_0 + \beta_1 x_i)/[1 + \exp(\beta_0 + \beta_1 x_i)]$.

Since the substitution at a residue is independent of substitution elsewhere in the protein, the likelihood function of n observations is obtained as the product of individual probability

$$L(\beta_0,\beta_1) = \prod_{i=1}^{n} \theta^{y_i} (1 - \theta)^{1-y_i} \quad (3)$$

Estimates for β_0 and β_1 can then be obtained by maximizing the logarithm of the likelihood function with respect to β_0 and β_1.

Data Analysis

Our study included 68 proteins of class I PLA_2 enzymes. Each protein has about 118 amino acid residues. For each residue, the relative surface accessibility and whether a substitution has occurred were recorded. Hence there were 8024 pairs of observations altogether. Similarly, 59 proteins of class II PLA_2 enzymes, each with about 122 residues, resulted in 7198 pairs of observations. The procedure CATMOD (categorical data modeling) in SAS (Statistical Analysis System) was used to find the fitted logistic models for these two sets of data (Agresti 1996). Maximum-likelihood method was employed to find the estimates of the coefficients in the logistic models. We estimated coefficients, $\hat{\beta}_0$ and $\hat{\beta}_1$, of the fitted logistic model and the probabilities of substitution for buried ($x = 0$) and exposed ($x = 100$) residues. The ratio of the two estimated probabilities of substitution between buried and exposed residues was also calculated for both classes of PLA_2 enzymes.

Results and Discussion

Amino Acid Substitutions in PLA_2 Enzymes

We examined natural substitutions in both class I and class II snake venom PLA_2 enzymes (Table 1) (Heinrikson et al. 1977). As shown in Fig. 1, substitutions in 68 PLA_2 enzymes and their homologues from class I (from elapid and hydrophid snake venom) and 59 from class II (from crotalid and viperid snake venom), respectively, do not occur at random; there are several hot spots and several cold spots. We examined three-dimensional structures of PLA_2 enzymes to determine the parts of segments where substitutions of amino acids occur at a higher rate (Fig. 2). Generally, the residues that are substituted at a faster rate (between 40 and 80%) are located on the surface of the molecule, whereas the residues that are substituted at a slower rate (between 0 and 40%) are buried inside the molecule. Therefore we examined the relationship between surface accessibility of the amino acid residue and its substitution rate using a logistic

Table 1. Origin of venom phospholipases and their homologues

Genus	Geographic distribution	Number		
		Species	Subspecies	Proteins
Class I				
1. *Enhydrina*	Asia	1	1	2
2. *Aipysurus*	Australia	1	1	1
3. *Laticauda*	Asia, Australia	3	3	6
4. *Oxyuranus*	Australia	1	1	3
5. *Notechis*	Australia	1	1	7
6. *Pseudonaja*	Australia	1	1	3
7. *Pseudechis*	Australia	2	2	16
8. *Micrurus*	Middle East/Asia	1	1	1
9. *Hemachatus*	Africa	1	1	1
10. *Aspidelaps*	Africa	1	1	1
11. *Naja*	Africa, Asia	4	9	19
12. *Bungarus*	Asia	2	2	8
Class II				
1. *Vipera*	Asia, Europe	3	5	12
2. *Pseudocerastes*	Middle East	1	1	2
3. *Cerastes*	Asia	1	1	1
4. *Bitis*	Europe	1	1	1
5. *Eristocophis*		1	1	2
6. *Bothrops*	South & Central America	3	3	6
7. *Agkistrodon*	Asia, North America	4	5	9
8. *Trimeresurus*	Asia	4	4	19
9. *Crotalus*	North & South America	4	4	7

model. The results based on this model indicate that there is a significant correlation between these two parameters; surface residues are substituted at much higher rate than the buried residues in both class I and class II proteins (Table 2). The probability of a substitution in a fully exposed residue is about 3.5 and 2.6 times higher than a buried residue in class I and class II PLA_2 enzymes, respectively. Thus not only do natural mutations in the genes of PLA_2 enzymes occur more often in exons compared to introns (Ogawa et al. 1992; Nakashima et al. 1993, 1995; Nobuhisa et al. 1996), but also they appear to be predominant at the molecular surface of PLA_2 enzymes.

The database (Danse et al. 1997) used in this study consists of all the sequenced PLA_2 enzymes and their homologues independent of their PLA_2 enzymatic activity or toxicity. In several cases almost all the isoenzymes from a single snake venom have been sequenced (Nakashima et al. 1993; Takasaki et al. 1990b). Thus, the lower number of substitutions in cold spots is not a direct reflection of a skewed database, in which protein examples that have been eliminated during evolution (because of their severe detrimental effect on the snake) are not equally represented. In addition, PLA_2 enzymes considered in this study were isolated from the venom of snakes belonging to 21 genera from many geographic regions of the world (Table 1) and these snakes have different food habits. Thus, the amino acid substitution results reflect the overall tendency in the evolution of PLA_2 enzymes, and not geographic variations or diet (Jayanthi and Gowda 1988; Daltry et al. 1996).

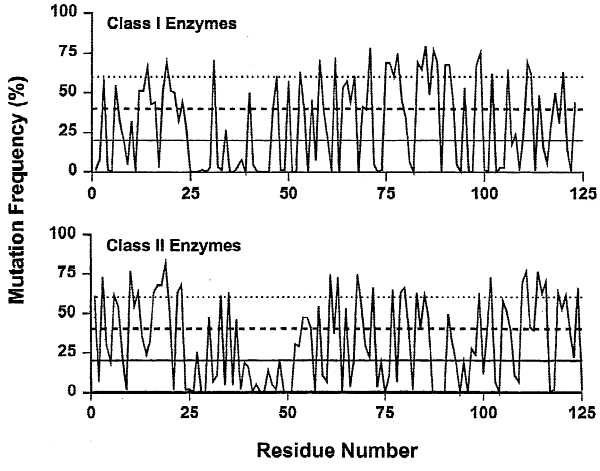

Fig. 1. Frequency of mutations in snake venom PLA_2 enzymes. Mutation frequencies were calculated based on the number of mutations in each amino acid position compared with that of the consensus sequence.

Minimal Selection Pressure on PLA_2 Genes

If a protein is crucial for the existence or survival of the organism, and if the mutation results in an inactive or less active form, such substitutions could be eliminated by selection pressures. The following factors indicate that there may not be significant amount of selection pressure on PLA_2 enzymes.

(1) The selection pressure is effective when there is only one copy of the protein. A mutation, which results in the loss of function, in this single copy could be det-

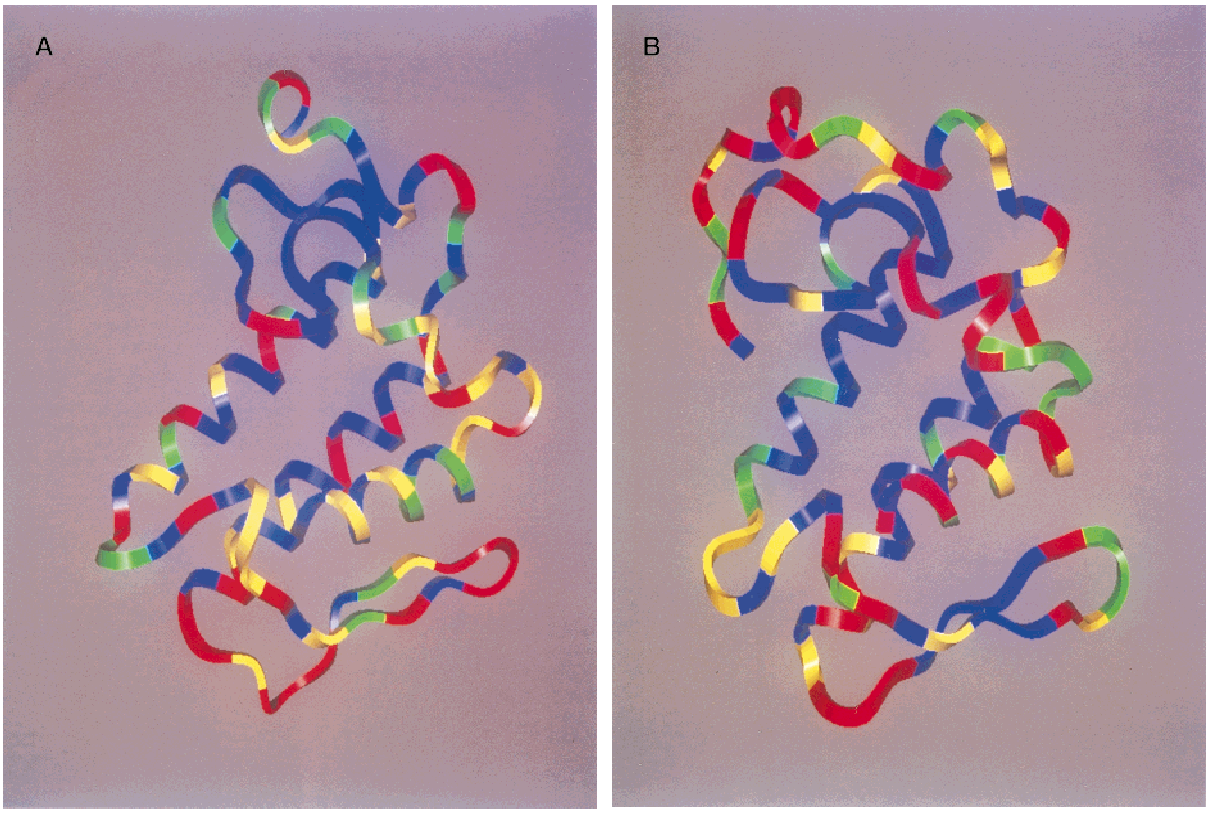

Fig. 2. Tertiary structure of PLA_2 enzymes showing mutational frequency. **A** Class I PLA_2 enzyme (White et al. 1990). **B** Class II PLA_2 enzyme (Brunie et al. 1985). The ribbon of the proteins is colored based on the mutation frequency of the residues; blue (0–20%), green (20–40%), yellow (40–60%), and red (60–80%).

Table 2. Relationship between surface accessibility and mutation rate

	$\hat{\beta}_1$	$\hat{\beta}_0$	$p_{(0)}$	$p_{(100)}$	$p_{(100)}/p_{(0)}$
Class I	0.0183 ± 0.000751	−1.7398 ± 0.0464	0.1493	0.5225	3.5
Class II	0.0152 ± 0.000826	−1.4579 ± 0.0481	0.1888	0.4845	2.6

rimental to the organism. When there are more copies, a mutation in one of the copies is not lethal (Dickerson and Geis 1969). Thus gene duplication and gene conversion play a critical role in gaining proteins with new functions. In general, several PLA_2 isoenzymes are found in an individual snake venom. Almost all snake venoms examined so far contain multiple PLA_2 isoenzymes, sometimes more than 10 (Braganca and Sambray 1967; Salach et al. 1971; Vishwanath et al. 1987; Takasaki et al. 1990a). Mutations and substitutions in such a family of genes are generally not lethal, as less efficient proteins can be carried as dead weight (Dickerson and Geis 1969).

(2) PLA_2 enzymes play an important but not a crucial role in the survival of the organism. Venom PLA_2 enzymes, like other venom proteins, are injected into the prey. They contribute toward procurement of food through immobilization and digestion of the prey (Tu 1977; Abe et al. 1977; Bon 1982). PLA_2 isoenzymes probably help in the immobilization through their inherent ability to induce various pharmacological effects (see below). If a specific PLA_2 enzyme mutates into an inactive protein, either a related PLA_2 isoenzyme or other non-PLA_2 toxins in venom could ''replace'' these functions, at least partially. Thus substitutions in PLA_2 proteins may not be severely detrimental to the snake. The substitutions in PLA_2 enzymes may, however, have some implications for food acquisition (through immobilization) and digestion, but this may not be severe enough to eliminate the individual snake.

(3) Accelerated evolution appears to be prevalent in most families of venom proteins. For example, accelerated evolution is also observed in venom serine proteinases (Deshimaru et al. 1996). Judging from their functional variability, other toxin families including three-finger toxins, c-type lectin-related proteins, and metalloproteinases are probably developing through accelerated evolution.

(4) There appears to be little preference for either higher toxicity or a specific pharmacological activity

during evolution of venom PLA_2 isoenzymes. PLA_2 isoenzymes show a wide range of toxicity to laboratory animals, ranging from highly toxic (1 μg/kg) to nontoxic (300 mg/kg) proteins (Rosenberg 1990). There is no correlation between the toxicity of these proteins and their abundance; in some cases toxic enzymes are abundant, whereas in others nontoxic enzymes are predominant.

These factors support our contention that there is only minimal selection pressure on the evolution of PLA_2 enzymes and ''invalid'' substitutions are not easily eliminated.

Mutations in Surface Residues and Evolution

Some of the common beliefs among scientists regarding molecular evolution of proteins are as follows (Creighton and Darby 1989): conserved residues in a group of related proteins are important for the biological function of the protein and the most variable residues are not. Amino acids in the protein core region are important for the protein folding (or conformation) and are changed less frequently than the surface residues. Therefore, most substitutions, insertions, and deletions occur on the surface, where they need not interfere in its structure (or conformation) or function (Creighton and Darby 1989). In the case of enzymes these beliefs are probably true; the active site is generally conserved and is located in an interior pocket. Any mutation in the protein core could lead to the loss of catalytic efficiency, the inherent biological function of the enzyme protein. In these cases, mutations on the surface of the protein need not have significant effects on the enzymatic activity. However, in the case of ligands and receptors, surface residues play critical role in protein–protein interaction and hence their function. In these cases, mutations in the surface residues could alter the function of the protein. Venom PLA_2 enzymes, like other toxins, interact with receptor/acceptor proteins. Therefore, the surface residues are important in molecular recognition. Natural substitutions in the surface residues would hence play a significant role in their evolution.

Substitutions in the surface residues are also recognized to play an important role in the evolution of other classes of proteins. A recent parsimony analysis of the protein-coding regions of the mitochondrial genome of 19 taxa resulted in incorrect placements in the phylogenetic tree (Naylor and Brown 1997). The authors examined details of the structural aspects of the sites and their contribution to phylogenetic estimation; sites coding for hydrophilic amino acid residues produced better fits than those coding for hydrophobic residues (Naylor and Brown 1997). It is important to note that the majority of hydrophobic residues are found in the ''core'' of the protein and not on the surface, whereas hydrophilic residues are generally found on the surface. Thus, substitutions do not occur as often in the hydrophobic core of the proteins during evolution and hence using the hydrophobic residues can result in misleading alignments in phylogenetic analysis.

Accelerated Evolution and Pharmacological Effects

PLA_2 enzymes induce a wide variety of pharmacological effects, including neurotoxic, cardiotoxic, myotoxic, anticoagulant, antiplatelet, edema-inducing, hemolytic, and hemorrhagic effects (Kini 1997). Previously, we proposed a hypothetical model to explain how and why PLA_2 enzymes induce various pharmacological effects (Kini and Evans 1989). According to the model, PLA_2 enzymes ''target'' themselves to a specific organ or tissue, via their high-affinity binding to specific proteins. Upon binding to the target protein (or glycoprotein), they induce the effects either dependent on or independent of their enzymatic activity. In recent years considerable experimental evidence has been accumulated in support of this model (Lambeau et al. 1989, 1997; Stefansson et al. 1990; Yen and Tzeng 1991; Krizaj et al. 1994). The specific binding of PLA_2 to its protein target is conferred by the presence of a ''pharmacological site'' on its surface which is independent of the catalytic site (Condrea et al. 1981; Rosenberg 1986; Kini and Evans 1987, 1989). As shown here, the natural substitution in this group of proteins occurs predominantly in surface residues and hence contributes directly toward modifying the molecular surface. Thus through the accelerated evolution (Ogawa et al. 1992; Nakashima et al. 1993, 1995; Nobuhisa et al. 1996) and natural substitutions, nature appears to experiment with and modify the molecular surface to afford distinct and novel targeting to cells or tissues. This is not surprising since PLA_2 toxins, like other groups of toxins in snakes (discussed earlier) and scorpions (Menez 1993; Menez and Dauplais 1997; Menez et al. 1992), are based on a simple molecular mold or template. These molecular templates have been used to develop variety of toxins that are capable of attacking different physiological systems. Different functional sites have also evolved in the other groups of small toxin polypeptides through substitutions (Menez 1993; Menez and Dauplais 1997; Menez et al. 1992). However, most of these molecules are too small and hence difficult to analyze and assess the relationships between substitution and surface accessibility of the amino acid residues.

Neutral Versus Selection Theories of Molecular Evolution

In nature, a great majority of mutations or substitutions are lost within a few generations. According to the neutral theory of molecular evolution (Kimura 1968; King and Jukes 1969), the majority of mutations that are fixed in a population are selectively neutral. These neutral mutations have little or no effect on the function of the gene

or its protein product and they cause genetic drifts in a population. Thus this theory suggests non-Darwinian evolution of molecules. As discussed above, PLA_2 enzymes induce distinctly different pharmacological effects and show a wide range of toxicity and catalytic efficiency in hydrolyzing phospholipids. Thus both catalytic and functional properties of PLA_2 enzymes are altered during evolution. Thus these natural substitutions in PLA_2 enzymes are not neutral or near-neutral (Ohta and Kreitman 1996).

At the molecular level, mutations that alter the structure and function of the gene or its product are selected against, rather than selected for (Creighton and Darby 1989). Thus deleterious mutations are weeded out through negative Darwinian selection. This is particularly effective on single-copy genes and when these genes play vital roles in the survival of the organism. As described above, evolution of PLA_2 enzymes is probably under minimal pressure.

Positive Darwinian selection is rare but appears to occur in a small number of proteins. Functional divergence of proteinases and their protein inhibitors provides an example of this type of evolution (Hill and Hastie 1987; Laskowski et al. 1987; Brown 1987; Creighton and Darby 1989; Ohta 1994). Specific and controlled proteolysis is essential in various physiological processes. Highly specific proteinases and their inhibitors monitor and regulate various physiologic systems. The fidelity in substrate specificity of a proteinase is due to a small number of amino acid residues that contribute to the active site. Similarly a short segment of proteinase inhibitor interacts with the proteinase. This protein–protein interaction segment, called the reactive site, contains the susceptible peptide bond. Structural analyses of both active sites of proteinases and reactive sites of proteinase inhibitors indicated that the amino acid residues in these sites are substituted more often than other nonfunctional sites (Hill and Hastie 1987; Laskowski et al. 1987; Brown 1987; Creighton and Darby 1989; Ohta 1994). Thus the specificity of proteinases and proteinase inhibitors is changing during their evolution. Both proteinases and the inhibitors are thought to be coevolving through positive Darwinian selection (Creighton and Darby 1989).

The case of proteinase inhibitors (Laskowski et al. 1987) is similar, if not identical, to venom PLA_2 enzymes. In both cases, substitutions in the functional sites, reactive site residues, and surface residues in proteinase inhibitor and PLA_2 enzymes, respectively, could lead to change in functional specificity and not necessarily to a loss of function. The loss of original specificity due to substitution in the functional site a PLA_2 enzyme can be compensated by the presence of closely related isoenzymes. Akin to proteinase inhibitors, PLA_2 enzymes also target exogenous receptors (or acceptors) and hence the accelerated evolution in this class of proteins also has a purpose of modifying the molecular surface (see discussion above). Thus positive Darwinian selection in PLA_2 enzymes is, as Loskowski et al. (1987) elegantly described in the case of serine proteinase inhibitors, ''not only technologically possible and affordable, but there may be a reason for it.'' However, in contrast to the case of proteinase–protein inhibitor coevolution, the receptor/acceptor protein for venom PLA_2 enzymes may not be evolving at accelerated rates. This argument could be based on the fact that, in general, most of these receptor/acceptor proteins have critical functions in the prey.

Directed (or Advantageous) Mutations

In lower organisms, for example, in prokaryotes and virus, directed (or advantageous) mutations have been recognized (Cairns et al. 1987; Hall 1990; LeClerc et al. 1996; Brown and Richman 1997). In these cases, directed mutations preferentially occur in cells in an environment (or conditions) of stress, such as prolonged starvation of specific nutrients or carbon sources and antibiotic treatment. Such advantageous mutations relieve the immediate stress. There was no significant increase in mutation rates at other loci in these organisms (Hall 1990). All the above studies examined the mutation rates in several structurally and functionally unrelated genes (that are not derived by a single ancestral gene) and reflect the population dynamics of single-cell organisms (prokaryotes) and viruses. It is not clear whether natural substitutions in the surface residues of venom PLA_2 enzymes are in fact directed (or advantageous) mutations. Apparently these substitutions are advantageous to snakes in developing new toxins that target different tissues.

Conclusions

Gene duplication, accelerated evolution, gene conversion, and positive Darwinian selection contribute to molecular evolution of PLA_2 enzymes. Natural substitutions appear to occur predominantly on the molecular surface of PLA_2 enzymes. These substitutions change the specificity of PLA_2 enzymes in binding to their target tissues, enabling them to achieve novel targeting. Similar phenomena may also exist in other classes of toxins. By altering the molecular surface, nature has developed proteins with similar molecular folds but with multiple functions. Positive Darwinian selection may play a role in the evolution of venom PLA_2 enzymes.

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