The Electrostatic Basis for the Interfacial Binding of Secretory Phospholipases A_2

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ABSTRACT Biochemical and structural data suggest that electrostatic forces play a critical role in the binding of secretory phospholipases A_2 to substrate aggregates (micelles, vesicles, monolayers, and membranes). This initial binding (adsorption) of the enzyme to the interface is kinetically distinct from the subsequent binding of substrate to the buried active site. Thus, in the absence of specific active-site interactions, electrostatic forces operating at the molecular surface may orient and hold the enzyme at the interface. We have calculated the electrostatic potentials for 10 species of secretory phospholipases A_2 whose atomic coordinates have been determined by x-ray crystallography. Most of these enzymes show a marked electrostatic sidedness that is accentuated to a variable degree by the presence of the essential cofactor calcium ion. This asymmetry suggests a discrete interfacial binding region on the protein's surface, the location of which is in general agreement with proposals derived from the results of chemical modification, mutational, and crystallographic experiments.

INTRODUCTION

Phospholipases A_2 (PLA_2) specifically hydrolyze the 2-ester bond of 1,2-diacyl-3-*sn*-phosphoglycerides. The large family of small (14 kDa) homologous proteins derived from extracellular sources (e.g., exocrine pancreas, reptile and insect venoms, and human synovial fluid) possesses a distinct set of functional features: (1) high specific activities (V_m of up to 5000 IU), (2) alkaline pH optima, (3) full enzymatic activity only in the presence of organized lipid-water interfaces (micelles, monolayers, vesicles, or membranes), and (4) calcium dependence (Waite, 1987; Achari et al., 1987; Dennis, 1983; Verheij et al., 1981).

Secretory PLA_2 ($sPLA_2$) hydrolyze phospholipids that contain one of several naturally occurring polar head groups (e.g., choline, ethanolamine, inositol, serine, and glycerol). High affinity binding of $sPLA_2$ to substrate aggregates occurs without any significant deformation in the gross organization of the bilayer or leakage of vesicle-trapped components (Jain and Rogers, 1989). Because adsorption of the enzyme to the substrate interface can be kinetically distinguished from the binding of a phospholipid molecule to the catalytic site (Fig. 1), it is possible to analyze the qualities of an interface separately from the process of substrate selection and the chemistry of catalysis (Ghomashchi et al., 1991; Berg et al., 1991; Jain et al., 1991; Ramirez and Jain, 1991). A crucial role for electrostatic interactions in interfacial adsorption is likely given that the affinity of $sPLA_2$ for anionic bilayers is generally orders of magnitude larger than for zwitterionic bilayers regardless of the precise phospholipid composition of the bilayer (Volwerk et al., 1986; Berg et al., 1991). Anionic and cationic additives (e.g., detergents) to the substrate interface increase and decrease, respectively, the catalytic efficiency of several $sPLA_2$ species (Volwerk et al., 1986; Jain and Jahagirdar, 1985; Apitz-Castro et al., 1982; Jain and Cordes, 1973). The binding of calcium ion to $sPLA_2$ also increases the affinity for aggregated substrate in some, but not all, enzyme species (van den Bergh et al., 1989; Menashe et al., 1986; Wells, 1972). Indeed, the adsorption of $sPLA_2$ to highly anionic vesicles can be sufficiently strong to make the enzyme completely processive; that is, the enzyme remains attached to the vesicle until virtually all of the phospholipid in the outer leaflet is hydrolyzed.

The three-dimensional structures of several $sPLA_2$s have been determined both in the absence (Freemont et al., 1993;

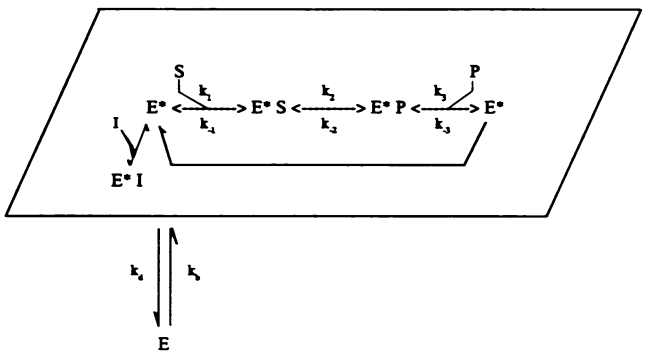

FIGURE 1 Schematic illustration of the scooting mode of catalysis by $sPLA_2$ (Berg et al., 1991). In this mechanism, which has substantial kinetic support (Ghomashchi et al., 1991; Berg et al., 1991; Jain et al., 1991; Ramirez and Jain, 1991; Jain and Rogers, 1989), adsorption of the enzyme (E) to the interface (E*) is a process that occurs before, and independent of, binding of substrate (S) to the active site (E*S). This permits the factors affecting adsorption and persistence of $sPLA_2$ at lipid-water interfaces to be selectively studied. (P = products, I = an inhibitor)

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TABLE 1 Selected crystallographic and biophysical characteristics of the phospholipases A_2 species included in this study

Phospholipase A_2 source	PLA_2 Class	pI	Aggregation state	Crystallization conditions	Charge groups
1. Porcine pancreas	I	7.4	Monomeric	pH 7.5/(+) Ca^{2+}/Organic	−15/+16
2. Bovine pancreas - zymogen	I		Monomeric	pH 7.5/(+) Ca^{2+}/Organic	−15/+15
3. Bovine pancreas - enzyme	I	9.2	Monomeric	pH 7.5/(+) Ca^{2+}/Organic	−14/+14
4. *Naja naja atra* venom	I	5.2	Monomeric	pH 7.5/(+) Ca^{2+}/Organic	−10/+10
5. *Agkistridon piscivorus piscivorus* venom	II	6.5	Dimeric	pH 7.5/(−) Ca^{2+}/Organic pH 7.5/(+) Ca^{2}/Organic	−17/+16
6. *Agkistridon piscivorus piscivorus* venom	II	9.5	Monomeric	pH 7.5/(−) Ca^{2+}/Organic	−13/+21
7. *Crotalus atrox* venom	II	5.5	Dimeric	pH 7.5/(−) Ca^{2+}/High Salt	−18/+12
8. *Crotalus adamanteus* venom	II		Dimeric	pH 7.5/(−) Ca^{2+}/High Salt	−16/+14
9. Human nonpancreatic (synovial fluid)	II	>10.5	Monomeric	pH 7.5/(+) Ca^{2+}/High Salt	− 8/+27
10. *Apis mellifera* venom		>10.5	Monomeric	pH 7.5/(+) Ca^{2+}/High Salt	−16/+25

Class distinctions (I or II) are made based upon the arrangement of disulfide bonds within the protein (Randolph et al., 1980). The bee venom PLA_2 is evolutionarily divergent but retains many of the core features of Class I/II enzymes (Scott et al., 1990b). The refined coordinates of these PLA_2 were derived from crystals obtained from a variety of solvent systems; however, it is unlikely that these distinctions, other than the presence or absence of calcium ion, are important to the electrostatic properties. "Charged residues" refers to a simple tabulation of the potential sources of negative charge [Asp, Glu] and the potential sources of positive charge [His, Arg, Lys].

TABLE 2 Amino acid sequences for the phospholipases A_2 included in this study

```
                1       5         10        15        20        25     ■29 ■   ■
Porcine pancreas  A L W Q F R S M I K C A I P G S H P L M D F N N Y G C Y C G L G G S
Bovine pancreas   * * * * * N G * * * * K * * S * E * * L * * * * * * * * * * * * *
Naja naja atra    N * Y * * K N * * Q * T V * * - * R S W W * * A D * * * * * * R * * *
App-dimer         D * M * * E T L * M K I A K - R S G M F W Y S A * * * * * * W * * Q
App-D49           N * F * * E K L * * K M T G - K S G M L W Y S A * * * * * * W * * Q
C. atrox          S * V * * E T L * M K I A G - R S G * L W Y S A * * * * * * W * * H
C. adamanteus     S * V * * E T L * M K V A K - R S G * L W Y S A * * * * * * W * * H
Human nonpanc.    N * V N * H R * * * L T T G - K E A A L S Y G F * * * H * * V * * R

                35        40        45    ○ ■ 50  □    55        60        65
Porcine pancreas  G T P V D E L D R C C E T H D N C Y R D A K N L D S C K F L V D N P Y
Bovine pancreas   * * * * * D * * * * * Q * * * * * * * K Q * * K * D * * * V * * * * * *
Naja naja atra    * * * * * D * * * * * Q V * * * * * * N E * E K I S G * - - - - - - W * *
App-dimer         * R * Q * A T * * * * F V * * C * * G K V T - - G - * - - - - - - D * K
App-D49           * R * K * A T * * * * F V * * C * * G K V T - - G - * - - - - - - * * K
C. atrox          * L * Q * A T * * * * F V * * C * * G K * T - - * - * - - - - - - * * K
C. adamanteus     * R * Q * A T * * * * F V * * C * * G K * T - - N - * - - - - - - * * K
Human nonpanc.    * K * K * * * * * * * * * * * * * * * * * * - - * - * - - - - - - * * *

                70        75        80        85        90        95       □100
Porcine pancreas  T E S Y S Y S C S N T E I T C N S K N N A C E A F I C N C D R N A A I
Bovine pancreas   * N N * * * * * * * N * * * * S * E * * * * * * * * * * * * * * * * *
Naja naja atra    F K T * * * * * * Q G T L * * K G G * * * * A * A V * D * * * L * * *
App-dimer         L D S * T * * V E * G D V V * G G - * * P * K K E * * E * * * A * * *
App-D49           M D I * T * * V E * G N * V * G G - T * P * K K Q * * E * * * A * * *
C. atrox          * V I * T * * E E * G * * I * G G - D D P * G T Q * * E * * K A * * *
C. adamanteus     * V I * T * * E E * G * * V * G G - D D P * G T Q * * E * * K A * * *
Human nonpanc.    * G K * * * * * W Q * N * * * * * - D D * * D * * * * * * * * * * *

                105       110       115       120       125       130
Porcine pancreas  C F S K A - - P Y N K E H K N L D T K - K Y C
Bovine pancreas   * * * * V - - * * * * * * * * * * * - * N *
Naja naja atra    * * A G * - - * * * N D N Y * I N L * - A R *
App-dimer         * * R D N K V T * D N K - Y W R F P P - Q N * K E E S E P C
App-D49           * * R D N L K T * D S K - T Y W K Y P K * N * * * * * * * * *
C. atrox          * * R D N I P S * D S K - T W L S P P - * D * R * * P * * *
C. adamanteus     * * R D N I P S * D N K - T W L S P P - * D * R Q * P * * *
Human nonpanc.    * * * * * L D * * * R N K * * * * * A - S * * Q * D * * * *

                1       5     ■ 9 ■   ■   15        20        25      29        ○
Apis mellifera    I I Y P G T L W C G H G N K S S G P N E L G R F K H T D A C C R T H
                35        40        45        50        55        60      ■ 65
                D M C P D V M S A G E S K H G L T N T A S H T R L S C D C D D K F Y D
                70        75        80        85  □     90        95       100
                C L K N S A D T I S S Y F V G K M Y F N L I D T K C Y K L E H P V T G
                105       110       115       120       125       130     134
                C G E R T E G R C L H Y T V D K S K P K V Y Q W F D L R K Y
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Asterisks are used to identify residues that are homologous to the prototype sequence (porcine pancreatic). The numbering system used is based upon the homologous core developed by Renetseder et al., 1985. Letters of residues bearing a potential positive charge (His, Arg, and Lys) or negative charge (Asp, Glu) are *italic* and **boldface**, respectively. Black rectangles appear above residues involved in binding the primary calcium ion; open rectangles appear above conserved residues of the catalytic network, and His48 appears with an open circle. The references for these sequences are porcine pancreatic (Puijk et al., 1977), bovine pancreatic (Fleer et al., 1978), *Naja naja atra* (Tsai et al., 1981), *A. piscivorus piscivorus* (App) D49 monomer (Maragnore and Heinrikson, 1993) and D49 dimer (Heinrikson, 1991), *C. atrox* (Randolph and Heinrikson, 1982), *C. adamanteus* (Heinrikson et al., 1977), and human nonpancreatic (Seilhamer et al., 1989). The sequence of the App-D49 monomer differs at position 78 (Asp→Glu) from the cited reference based on the interpretation of the high resolution (1.5 Å) crystal structure. The bee-venom PLA_2 sequence (Kuchler et al., 1989) is not aligned because of its low overall homology.

Figure 2(a)

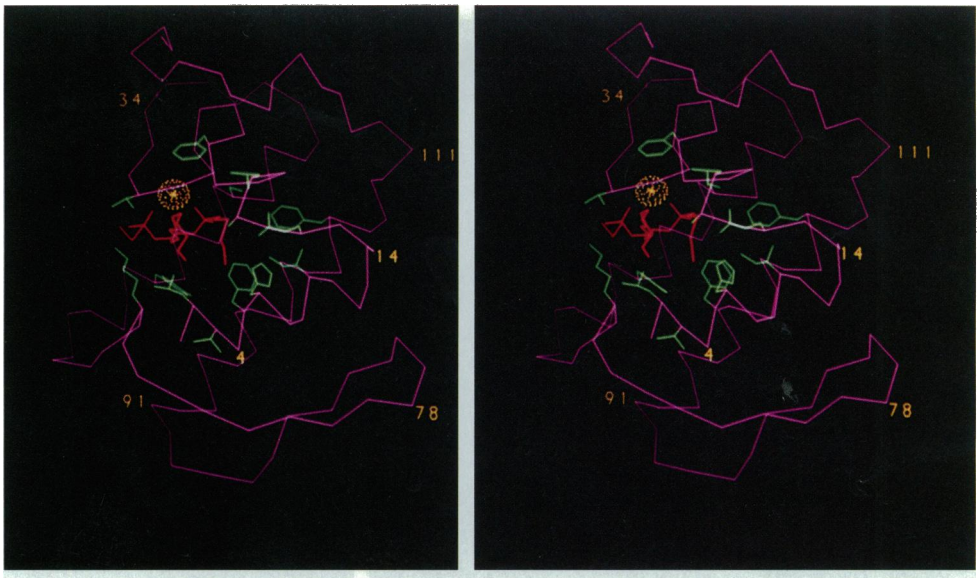


Figure 2(b)

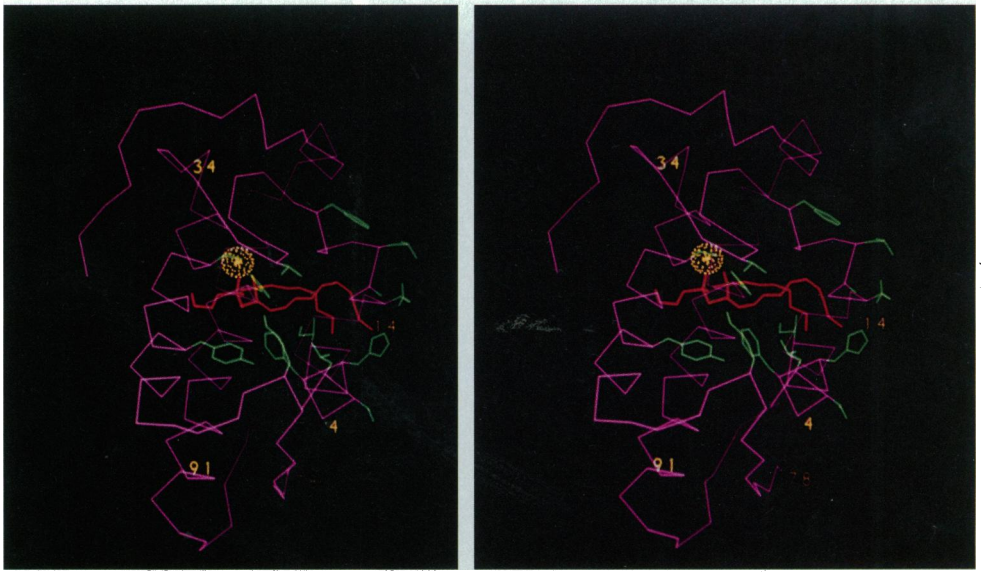
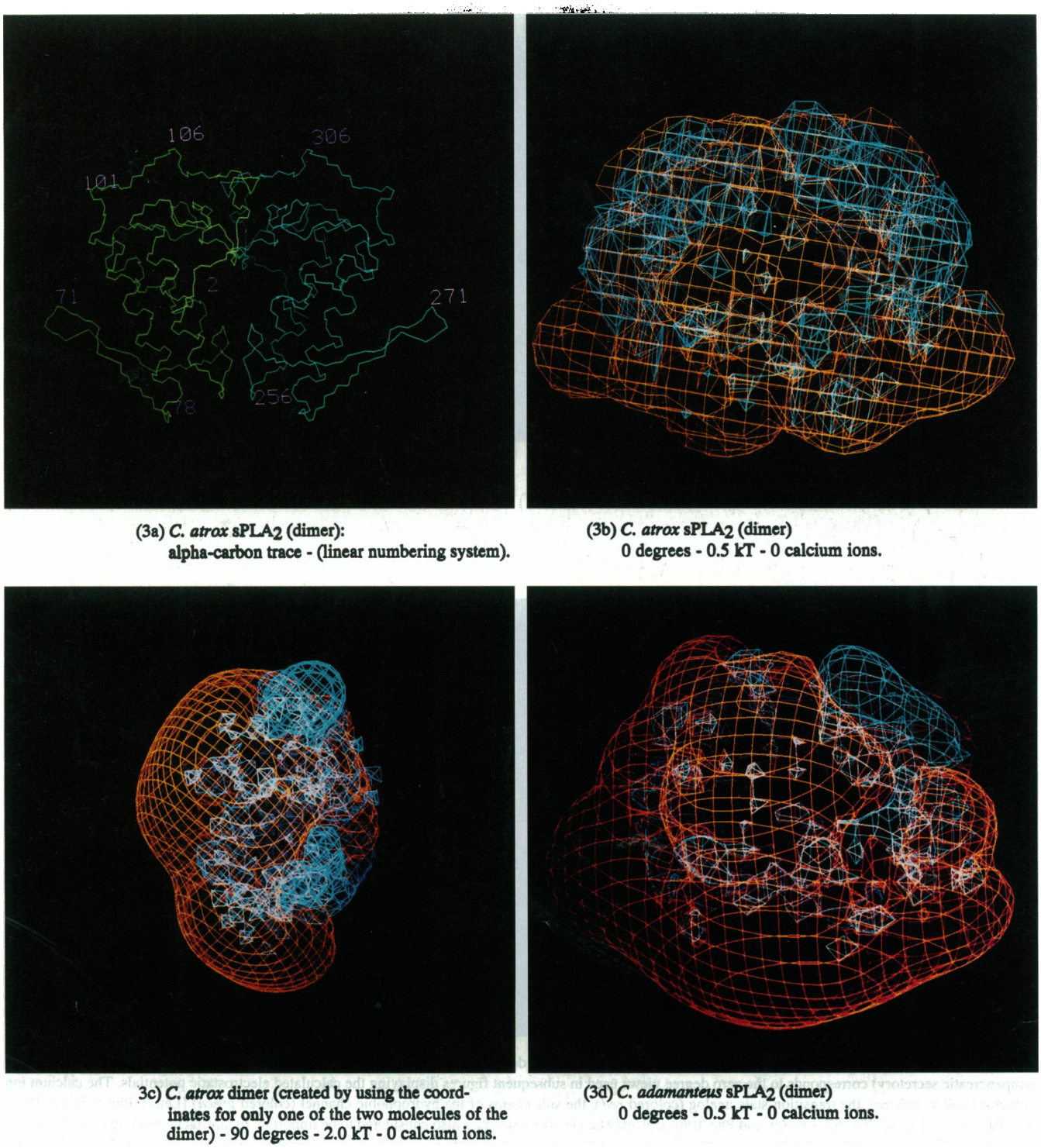


FIGURE 2 (*a*) Stereopair of the proposed interfacial binding surface—"0 degrees." The orientation of the C_α trace of this typical ClassI/II PLA_2 (human nonpancreatic secretory) corresponds to the zero degree views used in subsequent figures displaying the calculated electrostatic potentials. The calcium ion cofactor (*yellow sphere*), the transition-state analog (colored *red*), the side chains of the hydrophobic channel (colored *green*) [Leu-2, Phe-5, His-6, Ile-9, Ala-19, Val-31, Cys-45, Tyr-52, Lys-69, and Phe-106], and additional surface residues also considered to be important to interfacial binding [Val-3, Glu-17, Leu-20, and Phe-24] are shown. (*b*) Stereopair of the proposed interfacial binding surface—"90 degrees." The molecule shown in *a* has been rotated 90° counter-clockwise (as viewed from above) around a vertical axis that lies in the plane of the figure.

Westerlund et al., 1992; Wery et al., 1991; Scott et al., 1990a; Brunie et al., 1985; Dijkstra et al., 1983, 1982, 1981) and presence of transition-state (Scott et al., 1992; White et al., 1991; Scott et al., 1991b) and substrate analogs (Thunnissen et al., 1990). These structures, along with data from mutational and chemical modification studies (Yang and Chang, 1989; van Oort et al., 1985; Yoshida et al., 1988; de Haas et al., 1987; Dijkstra et al., 1981b), have implicated a discrete surface of the protein in the process of interfacial adsorption. This surface consists of a ring of positively charged side chains surrounding a hydrophobic channel that leads to the centrally located active site (Scott et al., 1990a). The binding, orientation, and persistence of $sPLA_2$ at the lipid-water interface is thought to be governed by the electrostatic and hydrophobic forces operative at this surface.



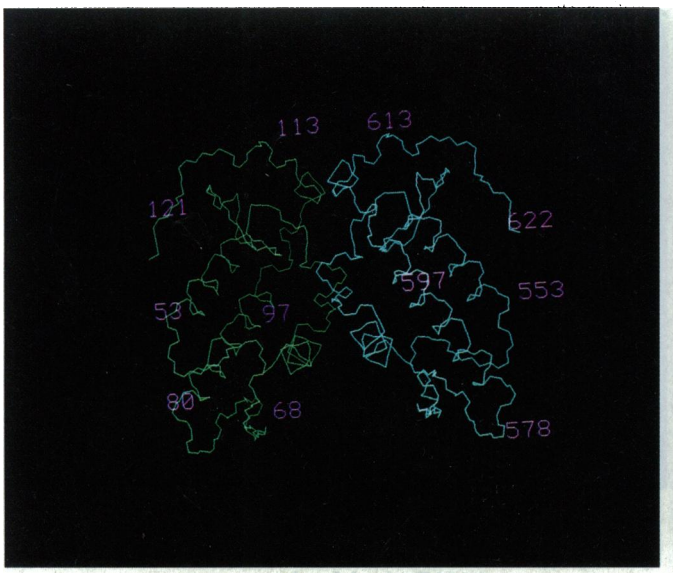
(3a) *C. atrox* sPLA2 (dimer): alpha-carbon trace - (linear numbering system).

(3b) *C. atrox* sPLA2 (dimer) 0 degrees - 0.5 kT - 0 calcium ions.

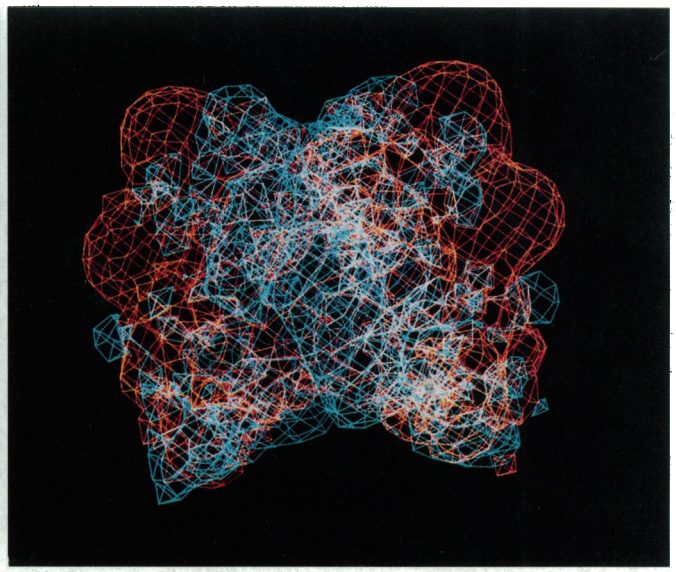
3c) *C. atrox* dimer (created by using the coordinates for only one of the two molecules of the dimer) - 90 degrees - 2.0 kT - 0 calcium ions.

(3d) *C. adamanteus* sPLA2 (dimer) 0 degrees - 0.5 kT - 0 calcium ions.

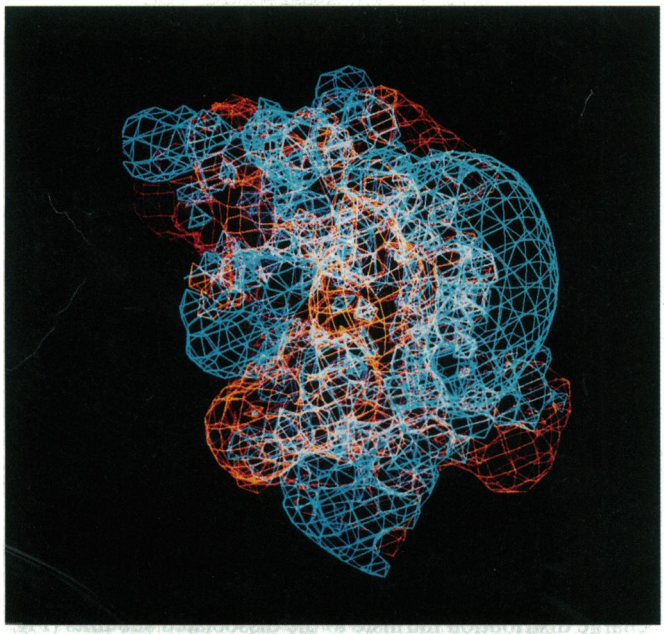
FIGURE 3 Electrostatic potentials calculated from the coordinates of crystalline phospholipases A_2: category I. Figures depict the electrical potentials of one or both standard orientations (0°/90°). An electrostatic potential of 1 kT/*e* (where *k* is the Boltzmann constant and *T* the is Kelvin temperature) is equal to 25 mV at 300 Kelvin. The contour levels of the maps were chosen to provide the maximum pictorial information possible about the respective electrostatic potential magnitudes and orientations. A contour level of 1 kT means that an atom of one unit of positive charge, sitting at a point on the contour grid, would have an interaction energy of +1kT (approximately +0.6 kCal/mole at 300 Kelvin) with the electrostatic potential at that point. Blue is used to indicate positive potentials, and red is used to indicate negative potentials. In three cases (the *A. p. piscivorus* and *C. atrox* dimers, and the bee-venom $sPLA_2$), additional skeletal traces are provided to clarify the molecular orientation. a. *C. atrox* $sPLA_2$ (dimer): alpha-cabbon trace—(linear numbering system). b. *C. atrox* $sPLA_2$ (dimer): 0 degrees—0.5 kT—0 calcim ions. c. *C. atrox* $sPLA_2$ (monomer created by using only one of the two molecules of the dimer): 90 degrees—2.0 kT—0 calcium ions. d. *C. adamanteus* $sPLA_2$ (dimer): 0 degrees—0.5 kT—0 calcium ions. e. *A. p. piscivorus* $sPLA_2$ (dimer): alpha-carbon trace—(linear numbering system). f. *A. p. piscivorus* $sPLA_2$ (dimer): 0 degrees—1.0 kT—0 calcium ions. g. *A. p. piscivorus* $sPLA_2$ (monomer created by using only one of the two molecules of the dimer): 0 degrees—1.0 kT— 0 calcium ions. h. *Apis mellifera* $sPLA_2$: alpha-carbon trace—(linear numbering system). i. *Apis mellifera* $sPLA_2$ (the glycosyl group of Asn13 was not included in the electrostatics calculations): 0 degrees—0.25 kT—1 calcium ion.



(3e) *A. p. piscivorus* dimer: alpha-carbon trace - (linear-numbering system).



(3f) *A. p. piscivorus* dimer 0 degrees - 1.0 kT - 0 calcium ions.



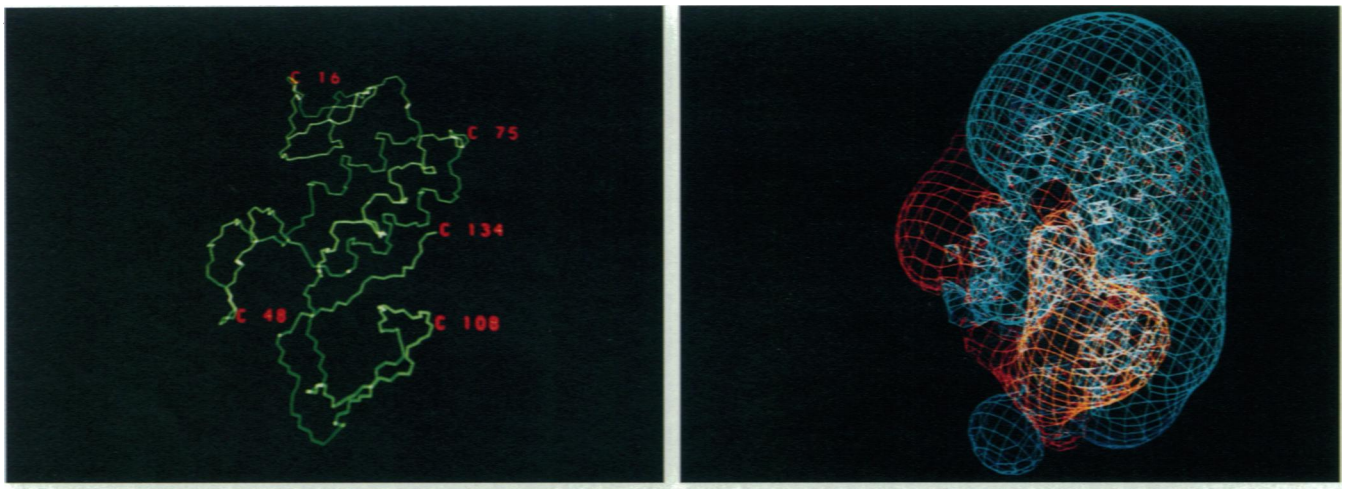
(3g) *A. p. piscivorus* dimer (created by using the coordinates for only one of the two subunits of the dimer) 0 degrees - 1.0 kT - 0 calcium ions.

FIGURE 3—*continued*

In this paper, we examined the surface electrostatic potentials of a diverse group of $sPLA_2$ that includes members of both Class I and Class II $sPLA_2$, the pancreatic prophospholipase (Class I), as well as the evolutionarily distant bee-venom enzyme. The electrostatic potentials calculated for these enzymes demonstrate a marked molecular sidedness, with the proposed interfacial binding surface generally lying in or adjacent to the most positively charged regions. This is consistent with the enhanced affinity and increased processivity exhibited by $sPLA_2$ on more negatively charged substrate aggregates. The essential, but weakly bound ($K_d > 10^{-4}$ M), calcium ion accentuates the protein's electrostatic sidedness and might play a particularly important functional role in those enzymes whose apo- state is only marginally asymmetric. In contrast, enzyme dimerization (Myatt et al., 1991) as seen in the $sPLA_2$ species from *Crotalus* and *Agkistridon* venoms confers no obvious electrostatic advantages.

MATERIALS AND METHODS

Coordinates for the $sPLA_2$s were obtained from either the Brookhaven Protein Data Bank or from recently completed studies at Yale. Secretory PLA_2 species were chosen for inclusion based on the availability of well



(3h) *Apis mellifera* sPLA$_2$: alpha-carbon trace - (linear numbering system).

(3i) *Apis mellifera* sPLA$_2$ (the glycosyl group of Asn13 was not included in the electrostatic calculations) - 0 degrees - 0.25 kT - 1 calcium ion.

FIGURE 3—*continued*

refined crystallographic coordinates, a suitable degree of biochemical and kinetic characterization, and the presence of unique structural/functional features (e.g., dimerization). The relevant biophysical properties and amino acid sequences are provided in Tables 1 and 2, respectively.

Electrostatic calculations were carried out using the DelPhi computer program (Nicholls and Honig, 1991; Klapper et al., 1986) and displayed using INSIGHT II (Biosym Technologies Inc., San Diego, CA). No corrections were made for the overestimation of electrostatic charge potentials by the linear Poisson-Boltzmann equation in the few cases where the potentials were very high. An ionic strength of 145 mM was chosen for the solvent region, and the temperature was set to 298 K for all calculations. The dielectric constant was set to 4 for the protein interior. The crystallographically observed water molecules were not treated explicitly but were instead included as part of the solvent region that was assigned a dielectric constant of 80 (Gilson et al., 1987; Sharp and Honig, 1990). In most cases, pK_a calculations were made for individual side chains and incorporated into the determination of electrical potentials. The effects of these calculations on the global results were, however, minor.

RESULTS

The high overall structural homology of these sPLA$_2$s permitted the choice of a single molecular orientation as the standard for comparison of electrostatic potential maps (Fig. 2). The view chosen as the standard ("0 degrees") is similar to that depicted in recent papers describing the structures of several sPLA$_2$ transition-state analog complexes (Scott et al., 1992, 1991a, b; White et al., 1991). The advantage of this view is that it looks directly down the hydrophobic channel while remaining perpendicular to the plane of the proposed interfacial binding surface. The calculated electrostatic potential maps (Figs. 3-5) are oriented according to this system. An additional view ("90 degrees" of rotation around the in-page vertical axis, Fig. 2 *b*) is provided where the standard view failed to illustrate electrical potential differences adequately between the front and back of the molecule.

DISCUSSION

The 10 sPLA$_2$ included in this study can be divided into three categories based on their calculated electrostatic potentials. Members of the first category, which includes the enzymes from the venoms of *C. atrox* (Western diamondback rattlesnake), *C. adamanteus* (Eastern diamondback rattlesnake), *A. p. piscivorus* (American cottonmouth water moccasin)-dimeric D49, and *Apis mellifera* (the honey bee), show distinct molecular sidedness in their distribution of positive and negative electrical potentials, but the scope and magnitude of the positive potential is relatively small (Fig. 3). The *C. atrox* sPLA$_2$ is the most striking in this regard, with the intact dimer having only a small patch of positive potential (Fig. 3 *b*). Calculation of the electrical potential for a theoretical monomer (only the structure of the crystallized dimer is known), however, reveals that the dimer interface quenches part of the charge distribution intrinsic to the dissociated subunits (Fig. 3 *c*). The magnitude and location of this quenched charge suggests that on the surface of a negatively charged substrate aggregate, the monomeric form is preferentially stabilized. An analogous prediction can be made for the dimeric enzyme from *A. p. piscivorus* venom where most of the positive electrostatic potential also resides at the shielded dimer interface (Fig. 3, *f* and *g*). The oligomerization state of the active enzyme at the interface remains controversial despite recent evidence supporting the monomer (Jain et al., 1991; Ferreira et al., 1993).

The second category of electrostatic distributions includes the enzymes from the venom of *Naja naja atra* (Taiwanese cobra) and from bovine pancreas (Fig. 4). In both of these sPLA$_2$, the most prominent positive electrostatic potential envelops a surface that is adjacent, but 45–90° away from the proposed interfacial binding surface (Scott et al., 1990a). The electrostatic asymmetry of this category of enzymes is quite

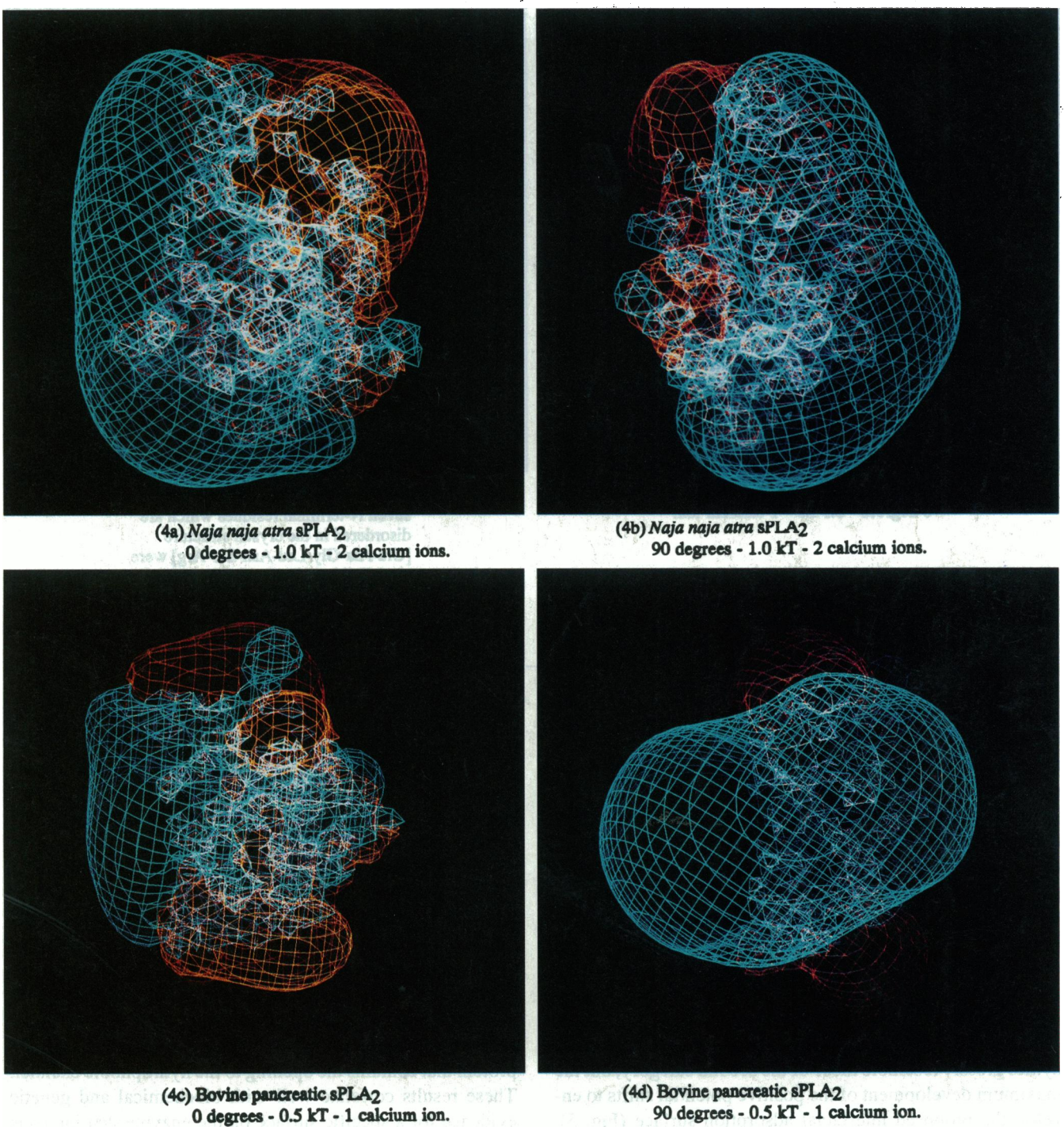
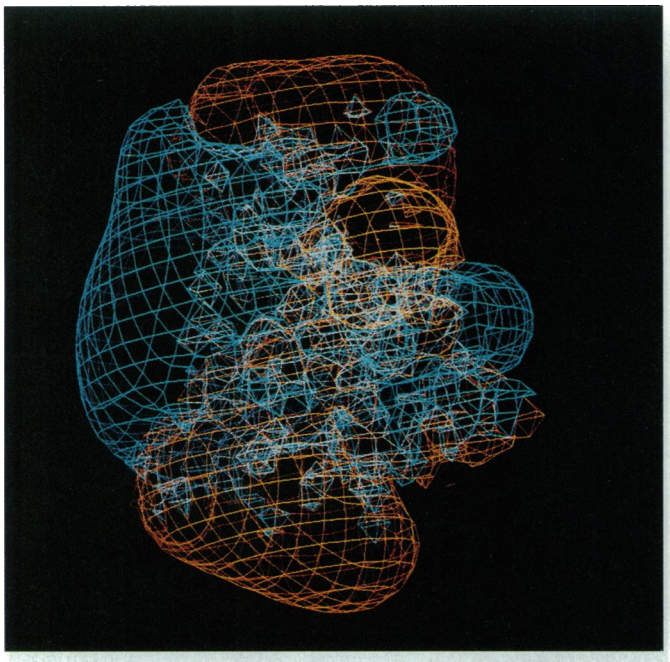
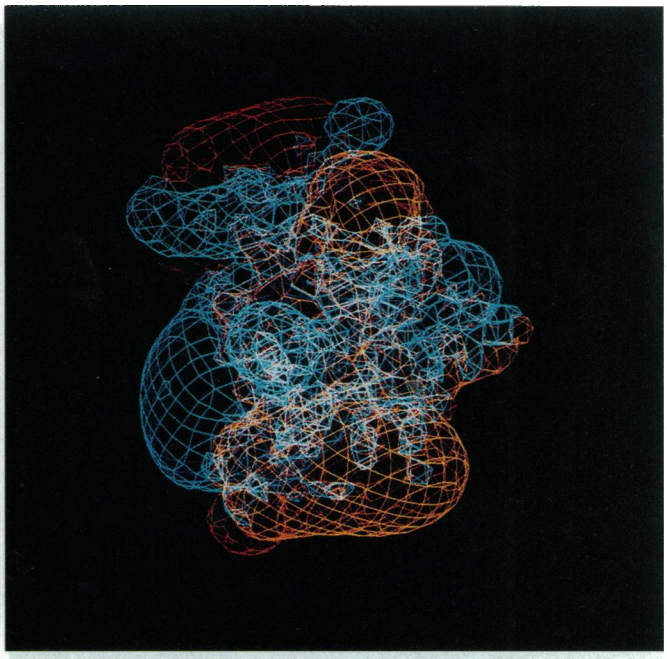


FIGURE 4 Electrostatic potentials calculated from the coordinates of crystalline phospholipases A_2: category II. Figures depict the electrical potentials of one or both standard orientations (0°/90°). An electrostatic potential of 1 kT/*e* (where *k* is the Boltzmann constant and *T* is the Kelvin temperature) is equal to 25 mV at 300 Kelvin. The contour levels of the maps were chosen to provide the maximal pictorial information possible about the respective electrostatic potential magnitudes and orientations. A contour level of 1 kT means that an atom of one unit of positive charge, sitting at a point on the contour grid, would have an interaction energy of +1 kT (approximately +0.6 kCal/mole at 300 Kelvin) with the electrostatic potential at that point. Blue is used to indicate positive potentials, and red is used to indicate negative potentials. a. *Naja naja atra* $sPLA_2$: 0 degrees—1.0 kT—2 calcium ions. b. *Naja naja atra* $sPLA_2$: 90 degrees—1.0 kT—2 calcium ions. c. Bovine pancreatic $sPLA_2$: 0 degrees—0.5 kT—1 calcium ion. d. Bovine pancreatic $sPLA_2$: 90 degrees—0.5 kT—1 calcium ion. e. Bovine pancreatic $sPLA_2$ (trans-aminated): 0 degrees—0.5 kT—1 calcium ion. f. Bovine pro-enzyme (the positions of the seven N-terminal residues that are disordered in the crystal structure [Glu-Ala-Gly-Leu-Asn-Ser-Arg] were modeled based on probable stereochemistry and packing considerations within the crystallographic unit cell): 0 degrees—0.5 kT—1 calcium ion.



(4e) Bovine pancreatic trans-aminated $sPLA_2$ 0 degrees - 0.5 kT - 1 calcium ion.



(4f) Bovine pro-enzyme (the positions of the seven N-terminal residues which are disordered in the crystal structure [Glu-Ala-Gly-Leu-Asn-Ser-Arg] were modelled based on probable stereo-chemistry and packing considerations within the crystallographic unit cell) 0 degrees - 0.5 kT - 1 calcium ion.

FIGURE 4—*continued*

distinctive, with obvious implications for the orientation of the molecule in its interactions with negatively charged substrate aggregates. The bovine pancreatic $sPLA_2$ is a particularly interesting case because the availability of the refined coordinates for the transamidated enzyme and the pro-enzyme permit the impact of an intact amino-terminus on the global electrostatics to be evaluated (Fig. 4 *c–f*). Modifications of the amino-terminus appear to only modestly alter the global electrostatic potentials. This argues against a simple electrostatic explanation for the failure of the transamidated and pro-enzymes to hydrolyze organized substrate aggregates efficiently (Jain et al., 1986).

The electrostatic potentials of the third category of enzymes grossly resemble those of the second category, but the maximum development of the positive potential shifts to envelop the proposed interfacial adsorption surface (Fig. 5). The three enzymes in this group, the D49 monomer from *A. p. piscivorus* venom, the human nonpancreatic secretory $sPLA_2$ (synovial fluid), and the porcine pancreatic enzyme, preserve a striking molecular asymmetry despite the predominance of positive electrical potential. In the case of the synovial $sPLA_2$, demonstration of this asymmetry requires the electrostatic maps to be displayed at very high contour levels (14 kT). The influence of the binding of the essential cofactor calcium ion on global charge distributions is particularly evident in the case of the porcine $sPLA_2$. The porcine enzyme has been shown by crystallography and by solution kinetics to bind two calcium ions per molecule. One of these ions participates as the essential cofactor in catalysis, the second calcium ion appears to be critical for the binding of the enzyme to aggregated substrate at alkaline pH (van den Bergh, 1989). From Fig. 5, *e* and *f* it is obvious that calcium ion markedly enhances the porcine enzyme's positive electrostatic potentials. A similar effect of the binding of calcium ion would be expected on the electrostatic potential distributions of other $sPLA_2$, especially for those enzymes with acidic isoelectric points (e.g., *C. atrox*).

In general, the electrostatic potentials calculated for these $sPLA_2$ show clear molecular sidedness with the maximum positive potential adjacent to or incorporating the face of the protein that contains the opening to the hydrophobic channel. These results correlate well with biochemical and genetic evidence for a specific surface on the enzyme that interacts with lipid aggregates (e.g., vesicles, monolayers, and membranes) (Jain and Vaz, 1987; Jain et al., 1986; Mao et al., 1986; Pieterson et al., 1974). The efficiency of this surface in promoting binding of the enzyme to the lipid-water interface is critically dependent on its charge composition (Ghomashchi et al., 1991; Berg et al., 1991; Jain et al., 1991; Ramirez and Jain, 1991; Thuren et al., 1987; de Haas et al., 1987, Volwerk et al., 1986). Spectroscopic experiments with the porcine pancreatic $sPLA_2$ have directly correlated the degree of interfacial adsorption with the mole fraction of the anionic component in the lipid aggregate. Highly anionic

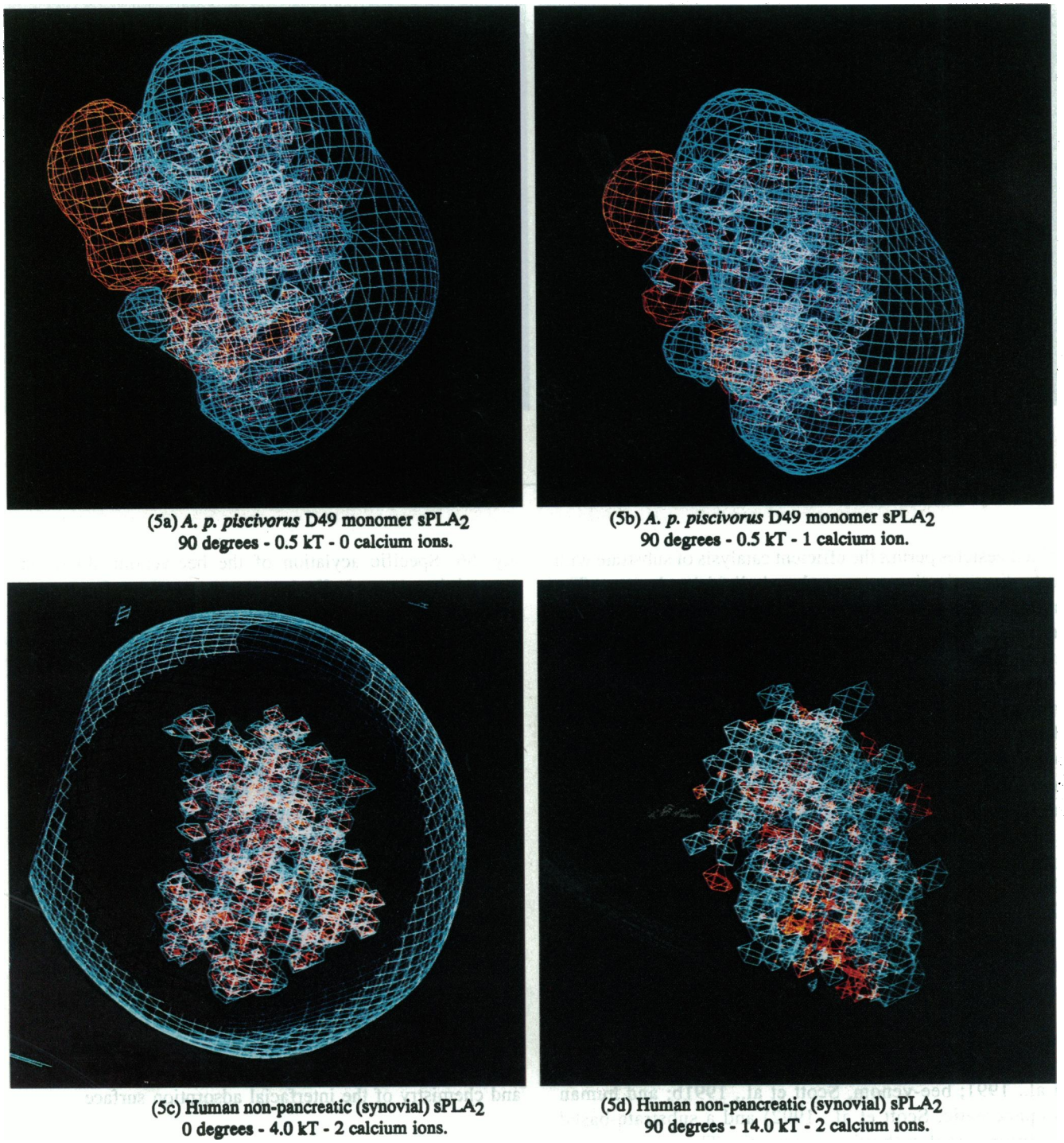


FIGURE 5 Electrostatic potentials calculated from the coordinates of crystalline phospholipases A_2: category III. Figures depict the electrical potentials of one or both standard orientations (0°/90°). An electrostatic potential of 1 kT/*e* (where *k* is the Boltzmann constant and *T* is the Kelvin temperature) is equal to 25 mV at 300 Kelvin. The contour levels of the maps were chosen to provide the maximal pictorial information possible about the respective electrostatic potential magnitudes and orientations. A contour level of 1 kT means that an atom of one unit of positive charge, sitting at a point on the contour grid, would have an interaction energy of +1 kT (approximately +0.6 kCal/mole at 300 Kelvin) with the electrostatic potential at that point. No correction was made for the over-estimation of electrostatic charge potentials by the linear Poisson-Boltzmann equation for *c* and *d*. Blue is used to indicate positive potentials, and red is used to indicate negative potentials. a. *A. p. piscivorus* $sPLA_2$ (49 monomer): 90 degrees—0.5 kT—0 calcium ions. b. *A. p. piscivorus* $sPLA_2$ (D49 monomer): 90 degrees—0.5 kT—1 calcium ion. c. Human nonpancreatic (synovial) $sPLA_2$: 0 degrees—4.0 kT—2 calcium ions. d. Human nonpancreatic (synovial) $sPLA_2$: 90 degrees—14.0 kT—2 calcium ions. e. Porcine pancreatic $sPLA_2$: 90 degrees—1.0 kT—0 calcium ions. f. Porcine pancreatic $sPLA_2$: 90 degrees—1.0 kT—2 calcium ions.

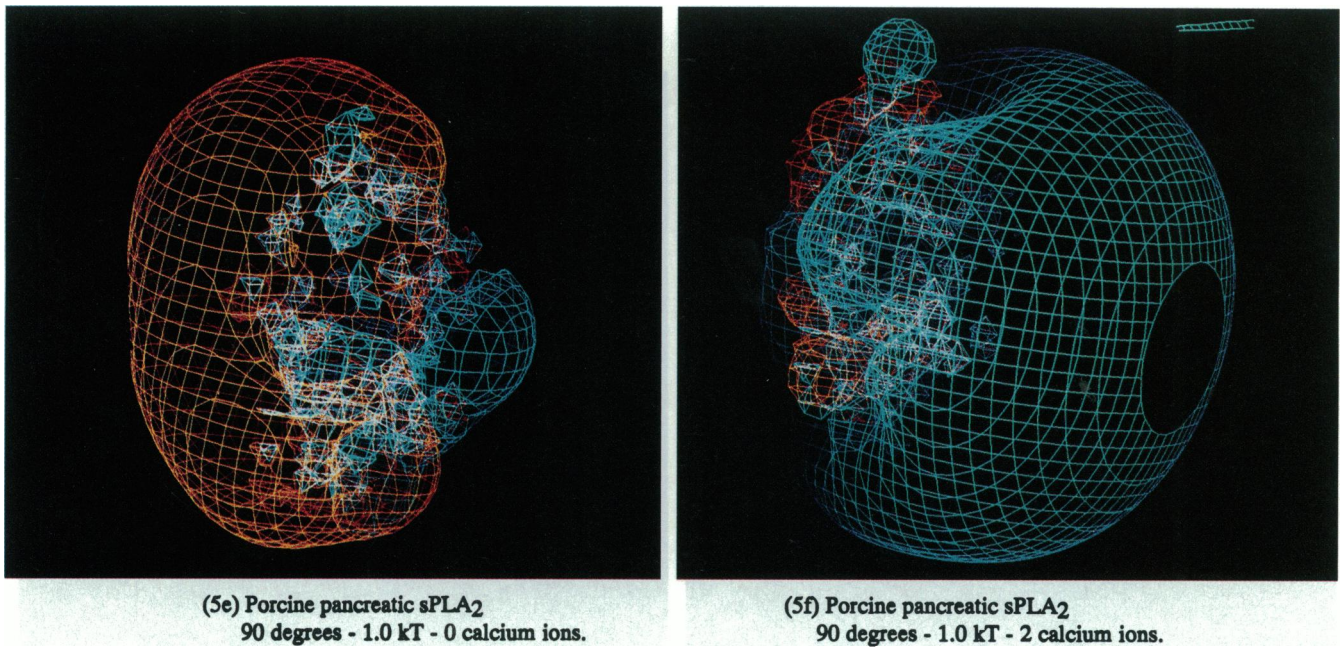


FIGURE 5—*continued*

mixed vesicles permit the efficient catalysis of substrate with little discrimination among phospholipid headgroups. The high efficiency of this "scooting mode" form of catalysis results, at least in part, from the persistence of the enzyme at the interface ($K_d < 10^{-13}$ M).

Mutational and chemical modification studies with the bovine pancreatic $sPLA_2$ suggested that the interfacial binding domain was a discrete protein face whose center lay at the opening to the hydrophobic channel (Fig. 2). Substitution of positively charged residues (e.g., arginine) for uncharged residues contributing to this surface (e.g., Asn-6) resulted in a two- to threefold higher value of K_{cat} on substrate aggregates (de Haas et al., 1987). Removal of sources of positive charge in this region either by specific mutations or by chemical modification of the protein lead to dramatic reductions in K_{cat}. Spectroscopic studies have correlated adsorption of the enzyme to the interface with the desolvation of uncharged residues forming the opening to the hydrophobic channel (Jain and Maliwal, 1993). The recently completed crystal structures of three $sPLA_2$ transition-state analog complexes (*Naja naja atra*, White et al., 1991; bee-venom, Scott et al., 1991b; and human nonpancreatic, Scott et al., 1992) and a substrate-based inhibitor complex (bovine pancreatic, Thunnissen et al., 1990) also clearly implicate this surface of the protein in interfacial adsorption.

Although the results described here are compatible with delocalized molecular electrostatics playing a key role in the orientation and persistence of $sPLA_2$ at water-lipid interfaces, there is also evidence for a strong hydrophobic effect (Tomasselli et al., 1989; Van der Weile et al., 1988; Cho et al., 1988; Drainas and Lawrence, 1978). Lugtigheid et al. (1993) observed a 60-fold increase in the apparent K_m of the porcine $sPLA_2$ for micellar substrate after acylation of Lys-56. Specific acylation of the bee-venom $sPLA_2$ improved the enzyme's K_{cat} by up to two orders of magnitude (Drainas and Lawrence, 1978). Presumably, the covalent attachment of hydrophobic groups activates $sPLA_2$ in a manner analogous to the addition of positive charge to the interfacial binding surface; i.e., increasing the affinity and persistence of the enzyme at the interface.

Clearly, the degree to which electrostatic forces affect interfacial catalysis can only be approximated by the techniques used here. Although an adequate description can be made of the enzyme's electrostatic field potentials, describing the environment at the surface of the bilayer in similar terms is at best difficult (Cevc, 1990). The 10 crystalline enzymes used in this study reveal a common, electrostatically favorable surface for potential interaction with substrate aggregates. Small variations in the location of this surface, such as seen in the Category II enzymes, suggest some flexibility in the initial adsorption that is presumably either optimized for catalysis by other forces or is inconsequential. Additional mutational and chemical modification studies should prove helpful in determining the precise boundaries and chemistry of the interfacial adsorption surface.

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