Circular dichroism analysis of ligand-induced conformational changes in protein kinase C

Mechanism of translocation of the enzyme from the cytosol to the membranes and its implications

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The structural changes following the binding to protein kinase C (PKC) of activators that promote its translocation to lipid environments were studied by far-u.v. c.d. and intrinsic fluorescence measurements of the protein. In the absence of activators, PKC contained 40 % α -helix, with an average size of 13 amino acids per α -helix segment, and 12 % β -structure as deduced from c.d. spectral analysis while fitting a set of model proteins of known structure. Ligands that promote translocation and activation of the enzyme, such as Ca^{2+} ions and phorbol esters, produced drastic changes in the c.d. spectra which may be

interpreted as a reduction in the average number of consecutive amino acids in the α -helix. Most of the total α -helix structure was conserved and an increase in β -structure was produced by active phorbol esters. These activators differentially affected the fluorescence of PKC: phorbol esters shifted the emission maximum to the red, whereas Ca^{2+} produced a marked decrease in the intensity of the fluorescence emission, suggesting in both cases that tryptophan residues were exposed to increased polar environments after binding of the ligands.

INTRODUCTION

Protein kinase C (PKC) was first described in neural tissues as an enzyme requiring phospholipids to express its full activity once activated by Ca^{2+} and diacylglycerol. At least eight different subspecies have been characterized after screening of cDNA libraries (Bell and Burns, 1991). Although these species exhibit important structural similarities, qualitative and quantitative differences have been observed concerning the specific requirements for maximal catalytic activity of each form (Nishizuka, 1986; Kikkawa and Nishizuka, 1986; Kikkawa et al., 1989). One of the relevant characteristics of PKC is its subcellular distribution. The soluble enzyme is inactive, whereas, once translocated to the membranes by Ca^{2+} , the enzyme constitutes a pre-activated state requiring only the transient generation of diacylglycerol to be active (Bell, 1986). For most of the agonist-mediated processes, in which PKC activation is involved, the increases in Ca^{2+} and diacylglycerol are elicited concomitantly (Berridge, 1987).

The distribution of PKC between the cytosol and the membranes is also controlled by the activators of the enzyme. The increase in the free cytosolic Ca^{2+} concentration that follows either mobilization from intracellular Ca^{2+} stores or entry of extracellular Ca^{2+} produces translocation of the soluble enzyme towards the membranes. However, the usual concentrations of Ca^{2+} , reached under physiological conditions, promote only a partial redistribution of the enzyme. In contrast, pharmacological analogues of diacylglycerols, such as phorbol diesters, trigger a rapid and complete translocation of the enzyme to the membranes, even in the absence of changes in cytosolic Ca^{2+} (Castagna et al., 1982; Kraft and Anderson, 1983; Nishizuka, 1984).

Although data are available on the minimal structural requirements for expression of PKC activity in the membrane-associated form (Rando and Young, 1984; Bazzi and Nelsestuen, 1991; Shah and Shipley, 1992), there are fewer on the mechanisms responsible for translocation of the enzyme from the cytosol to

the membranes. Phorbol diesters and Ca^{2+} are efficient in promoting PKC translocation to the membranes. The conformational changes elicited by binding of phorbols to PKC produce rapid reorganization of the protein structure which destabilizes the conformation of the protein in solution thereby favouring interaction with the membranes. The aim of this work is to determine whether the translocation and activation of PKC are related to the changes in secondary structure of the protein. One way to estimate the structural changes produced after ligand binding to a protein is to study its c.d. spectra in the far-u.v. region (Yang et al., 1986). In the present work we show that binding of both Ca^{2+} and β -phorbol 12,13-dibutyrate (PDBu) to soluble PKC markedly affects the conformation of the enzyme, producing a decrease in the average size of the α -helix structure. These changes are compatible with the increased ability of PKC to interact with the membranes.

MATERIALS AND METHODS

Chemicals

[γ - ^{32}P]ATP (3000 Ci/mol) was from New England Nuclear (Boston, MA, U.S.A.). Histone H1, phosphatidylserine (PS), dioctanoylglycerol, oleoylacetyl glycerol, phorbols, polylysine-agarose and protamine-agarose were from Sigma (St. Louis, MO, U.S.A.). DEAE-cellulose DR52 was from Whatman (Clifton, NJ, U.S.A.). Phenyl-Sepharose was from Pharmacia (Uppsala, Sweden). Nonidet P40 was from USB (Cleveland, OH, U.S.A.). Triton X-100 was from Packard. Acrylamide (electrophoresis grade) was from Bio-Rad (Richmond, CA, U.S.A.). Other chemicals were from Merck or Boehringer.

Purification of PKC

PKC was purified from bovine brain essentially as previously described (Woodgett and Hunter, 1987; Wooten et al., 1987). Bovine forebrains (300–400 g) were obtained from the local

abattoir and were immediately homogenized 1:4 (w/v). All procedures for the purification of the enzyme were carried out at 4°C to prevent proteolysis during the first steps of the purification. The homogenization buffer (buffer A) contained 4 mM EGTA, 4 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 % Nonidet P40, 2 % poly(ethylene glycol) (molecular mass 7 kDa), 10 µg/ml leupeptin, 1 mM phenylmethanesulphonyl fluoride, 100 units/ml Trasylol and 20 mM Hepes, pH 7.5. The homogenate was centrifuged at 50000 *g* for 30 min. The presence of poly(ethylene glycol) in the homogenization buffer sedimented the microsomal fraction, thus avoiding the necessity for centrifugation at higher speeds (105000 *g*). The purification was followed by DEAE-cellulose (DE52), phenyl-Sepharose, protamine-agarose and polylysine-agarose column chromatography (Woodgett and Hunter, 1987; Wooten et al., 1987).

The last step in the purification process was ultrafiltration (Ultrasart, Sartorius, Germany) through a cellulose triacetate membrane with a cut-off point of 30 kDa. The enzyme was diluted to 50 ml with a solution containing 20 mM KCl, 5 mM potassium phosphate, 1 mM dithiothreitol (DTT), 0.05 mM EGTA, 10 % glycerol and 20 mM Hepes, pH 7.5. The volume of the ultrafiltrate was reduced to about 5 ml and the last 5 ml excluded through the ultrafiltration membrane was used to analyse the contribution of the ligands to the c.d. spectra. The purity of the preparation was determined by SDS/PAGE (Laemmli, 1970). A unique band of 80 kDa was observed. The purification produced 2.3 mg of PKC with a yield of 14 %.

Lipid preparation

Lipids were dissolved in methyl chloride/methanol (19:1, v/v) and stored at -20°C under N₂. Vesicles containing PS and diacylglycerol were prepared after the lipids had been dried under a stream of N₂ and resuspended in the appropriate buffer followed by sonication at 4°C during four periods of 30 s at 80 % of the maximal intensity of an MSE sonicator. Mixed micelles containing Triton X-100 and different concentrations of PS and diacylglycerol were prepared as described by Hannun et al. (1985, 1986).

C.d. spectra

C.d. spectra were obtained with a Jobin Yvon mark III dichrograph provided with a 250 W xenon lamp at 20°C in a 0.05 mm path-length cuvette at a scanning speed of 0.5 nm/s, and a bandwidth of 0.5 nm, and each spectrum was the average result of three consecutive scans. The c.d. scans were recorded, the intensity of the signal being maintained below the transmission limit of the instrument. The solutions were filtered through a 0.4 µm pore filter (Dynagard, Microgon Inc.). To calculate the data, the contribution of the ligands and the medium to the c.d. spectra was subtracted from the corresponding spectra obtained in the presence of PKC. To minimize the differences between the spectra produced in the absence and presence of PKC, the excluded medium after ultrafiltration of the enzyme (cut-off 30 kDa) was used as a buffer to obtain the reference spectra. The experiments reported are the average of three to four different preparations of PKC and results are expressed as mean residue ellipticity (θ , degrees·cm²·dmol⁻¹) considering an average molecular mass residue of 110 Da.

Data analysis

The secondary structure was determined by the procedure of Menéndez-Arias et al. (1988) which treats the c.d. spectrum as a

vector of m elements, m being the number of measures of ellipticities at different wavelengths. The experimental spectra were compared with those of 18 proteins of known structure (Chang et al., 1978). The secondary structure of the protein was calculated by determining the best fit between the experimental c.d. spectra and a linear combination of reference spectra. This analysis provides the α -helix content, the average size of the α -helix sequences and the β -structure content (Chen et al., 1974; Provencher and Glöckner, 1981).

Fluorescence and absorbance analysis

The autofluorescence of PKC was recorded at 20°C by means of a Perkin-Elmer LS-50 spectrofluorimeter equipped with an Epson AT computer. Samples were excited at 295 nm and emission was recorded at 120 nm/min in the 305–400 nm region. The spectral bandwidth was 5 nm. Results are means of three successive scanings. The contributions of the ligands to the fluorescence spectrum were subtracted from the record in the presence of PKC.

Absorption spectra of PKC were produced at 20°C in a Shimadzu UV-2100 spectrophotometer.

Translocation of PKC *in vitro*

The *in vitro* translocation of purified brain PKC to PS vesicles was analysed by incubating the lipids and PKC with various amounts of phorbol esters. The assay buffer contained 10 mM 2-mercaptoethanol, 50 µM EGTA, 10 µg/ml leupeptin, 20 µg/ml vesicles, PKC, several concentrations of Ca²⁺ and PDBu, and 20 mM Hepes, pH 7.5. The translocation assay was carried out at 30°C for 5 min with continuous shaking (100 cycles/min). Samples of incubation mixtures (200 µl) were centrifuged at 105000 *g* for 30 min, and PKC activity was measured in the soluble and membrane-bound fractions after extraction with buffer A in the presence of 0.1 % Nonidet P40. All reagents used for these experiments were prepared in Chelex-treated water (Na⁺ form).

PKC activity was followed by phosphorylation of histone H1 in the absence and presence of Ca²⁺, PS and diacylglycerol as previously described (Woodgett and Hunter, 1987). To extract the vesicle-bound enzyme in the *in vitro* translocation assay, the pellet was resuspended in 2 ml of buffer A supplemented with 0.1 % Nonidet P40, followed by centrifugation (105000 *g* for 20 min), and processed as described for the soluble activity. One unit of PKC was defined as the incorporation of 1 µmol of phosphate into histone H1/min. Protein was determined as described by Bradford (1976) with BSA as standard.

RESULTS

Characterization of PKC

SDS/PAGE of the PKC preparation obtained by following the indicated purification protocol is shown in Figure 1. To ensure that this PKC retained its ability to be translocated from the soluble to the lipid environment, it was incubated in the presence of PS vesicles with either Ca²⁺ or Ca²⁺ plus PDBu, and the subcellular distribution of enzyme activity was analysed (Lee and Bell, 1986; Gopalakrishna et al., 1986). As Figure 2 shows, in the presence of 50 µM EGTA, the concentration of Ca²⁺ required to produce half-maximal translocation was 60 µM, but in the presence of 5 µM Ca²⁺, the concentration of PDBu required to produce half-maximal translocation was 55 nM. Moreover, in the absence of Ca²⁺ or PDBu, only 9 % of the enzyme was recovered in the lipid fraction. Hence this soluble PKC exhibited the expected behaviour of the cytosolic enzyme.

Figure 1 SDS/PAGE of purified bovine brain PKC

The enzyme was purified by the method of Woodgett and Hunter (1987) and Wooten et al. (1987). Protein (10 μ g) was analysed by SDS/PAGE (10% polyacrylamide gel) (lane 1) using as molecular markers β -galactosidase, BSA, egg albumin, glyceraldehyde 3-phosphate dehydrogenase and trypsinogen of molecular mass 116, 66, 45, 36 and 24 kDa respectively (lane 2).

Figure 2 Effect of Ca^{2+} and PDBu on binding of PKC to PS vesicles

Purified PKC (15 μ g/ml) was incubated at pH 7.5 in a medium containing 50 μ M EGTA, 10 μ g/ml leupeptin and 20 μ g/ml PS with various concentrations of Ca^{2+} (a) or 5 μ M Ca^{2+} and several concentrations of PDBu (b). After incubation at 30 $^{\circ}\text{C}$ for 5 min, the incubation mixtures were centrifuged at 105 000 g for 30 min and the PKC activity present in the supernatant was measured. Results are expressed as the percentage of the activity distributed between the soluble (●) and vesicle-bound fractions (○) (a) or as the percentage of the activity in the supernatant referred to the activity in the absence of PDBu (b).

Structure prediction deduced from the amino acid sequence

A knowledge of the primary structure of PKC allowed us to estimate the major organized elements of the secondary structure of the enzyme. We analysed the data on the complete amino acid sequence of bovine brain PKC determined by Parker et al. (1986) by the method of Gibrat et al. (1987) to predict the secondary structure from the sequence. Provided that at least four amino acids were present in each α -helix segment, the predicted structure contained 38% α -helix with an average size of 11 amino acids, 12% β -structure and 50% random coil. These data are compared with those obtained from c.d. spectral analysis of PKC.

C.d. analysis

The c.d. spectrum of purified bovine brain PKC is shown in Figure 3. Two separate minima exist at 221 and 210 nm. In this

Figure 3 Ligand-induced changes in the c.d. spectrum of soluble PKC

Purified PKC (180 μ g/ml) was incubated at 20 $^{\circ}\text{C}$ and pH 7.5 with: 50 μ M EGTA (A); 2 μ M PDBu (C); 100 μ M Ca^{2+} (D); 100 μ M Ca^{2+} plus 5 μ M non-active phorbol α -PDD (B); and 100 μ M Ca^{2+} plus 2 μ M PDBu (E). Data are shown as circles and the theoretical fits as continuous lines. The contributions of the ligands to the c.d. spectra were measured using the excluded medium after ultrafiltration of PKC, and was subtracted from the spectra in the presence of the enzyme. The structural motifs were calculated as described by Chang et al. (1978) and are given in Table 1.

Table 1 Effect of several ligands of PKC on the secondary-structure composition deduced from the c.d. spectra of the protein

Data were calculated from the c.d. spectra of Figures 3, 4 and 5 by the method of Chang et al. (1978). Results are means $\pm$ S.D.

Additions	Percentage of PKC as α -helix	Number of amino acids in α -helix
None	40	13 $\pm$ 2
PDBu (2 μ M)	38	11 $\pm$ 2
Ca^{2+} (100 μ M)	36	11 $\pm$ 2
Ca^{2+} (100 μ M) + α -PDD (5 μ M)	37	12 $\pm$ 2
Ca^{2+} (100 μ M) + PDBu (2 μ M)	28	7 $\pm$ 1
PS/diacylglycerol vesicles	35	10 $\pm$ 2
PS/diacylglycerol vesicles + Ca^{2+} (100 μ M)	21	7 $\pm$ 1
PS/diacylglycerol vesicles + PDBu (2 μ M) + Ca^{2+} (100 μ M)	20	7 $\pm$ 2
PKC at pH 4.5	21	8 $\pm$ 2

experiment, the enzyme was maintained in the presence of 50 μ M EGTA and in the absence of Ca^{2+} , and as shown in Figure 2, this structure may be assumed to correspond to that of the soluble enzyme. The spectra were processed as described by Menéndez-Arias et al. (1988), using a linear combination of reference spectra (Chen et al., 1974), and showed that PKC contains 40% α -helix structure with a predicted average size of 13 amino acids in each α -helix segment (Table 1). The compatibility of these results obtained from the c.d. spectrum of the protein with the secondary structure deduced from the amino acid sequence is remarkable.

PKC was incubated with 100 μ M Ca^{2+} , 2 μ M PDBu or 5 μ M

Figure 4 C.d. spectra of PKC in the presence of PS and diacylglycerol vesicles

Purified PKC (60 $\mu\text{g/ml}$) was incubated in the presence of: 50 μM EGTA (A); 50 $\mu\text{g/ml}$ PS and 2 $\mu\text{g/ml}$ diacylglycerol (B); 100 μM Ca^{2+} plus 50 $\mu\text{g/ml}$ PS and 2 $\mu\text{g/ml}$ diacylglycerol (C); and 100 μM Ca^{2+} , 2 μM PDBu, 50 $\mu\text{g/ml}$ PS and 2 $\mu\text{g/ml}$ diacylglycerol (D). The spectra were recorded at 20 $^{\circ}\text{C}$ and adjusted to model proteins as described in Figure 3. Experimental data are shown as circles and theoretical fits as continuous lines. The calculated data are given in Table 1.

Figure 5 C.d. spectra of PKC at pH 4.4

The protein was prepared in ultrafiltration medium containing 40 mM acetic acid/acetate and 20 mM Hepes, at either pH 7.5 (A) or pH 4.5 (B). The enzyme was incubated in the absence of ligands and was the same as reported in Figure 3. Experimental data are shown as circles and theoretical fits as continuous lines.

inactive phorbol 12,13-didecanoate (α -PDD) to analyse further the effect on the c.d. spectrum of ligands that promote the translocation of the enzyme from the cytosol to the membranes. Figure 3 shows the calculated c.d. spectra after subtraction of the contribution of the ligands. In these cases, the ligands were

incubated with the excluded medium after ultrafiltration of the enzyme solution through a 30 kDa membrane. In the presence of Ca^{2+} , in c.d. spectrum of the protein was slightly affected at both the 210 and 220 nm minima, reducing their contribution to the spectrum. The calculated structure (Table 1) showed no significant changes in α -helix content, but according to the prediction by the method of Chang et al. (1978), a small reduction in the content and size of these segments was evident. Also shown in Figure 3 is the effect of phorbol esters on the c.d. spectrum of PKC. In the presence of 100 μM Ca^{2+} , PDBu produced a drastic change in the c.d. spectrum, affecting the 221 nm minimum (Figure 3, spectrum E), which is clearly different from that either produced by Ca^{2+} (Figure 3, spectrum D) or PDBu alone (Figure 3, spectrum C). Owing to absorption of phorbols in the far-u.v. region, the c.d. spectra of samples containing this molecule cannot be recorded at wavelengths lower than 215 nm. The changes in the c.d. spectrum of PKC incubated with Ca^{2+} and PDBu suggest the existence of a synergism between the two ligands in promoting significant structural changes in PKC. However, the inactive phorbol isomer α -PDD produced only small changes in the spectrum obtained in the presence of Ca^{2+} (Figure 3, spectrum B). Calculation of these results by the method of Chang et al. (1978) suggested that Ca^{2+} and PDBu produce a significant decrease in content and size of the α -helix regions (Table 1), in agreement with the fact that both agents promote the translocation of PKC from the cytosol towards the membranes. Attempts to study the kinetics of the changes induced by both Ca^{2+} and PDBu in the c.d. spectra of PKC failed because of the rapidity of the process. Indeed, the changes in c.d. spectra produced by these ligands were stable at least during the 15 min period of scanning. Moreover, no significant differences were observed in the c.d. spectra recorded at protein concentrations in the range 40–400 μg of PKC/ml. To determine whether aggregation was involved in the mechanism of destabilization, PKC absorption spectra from 350 to 260 nm were obtained. No significant changes in the absorption profile were observed when the enzyme was incubated in either the absence or presence of Ca^{2+} and PDBu (results not shown).

The preceding results suggest that Ca^{2+} and PDBu, used at concentrations that promote the translocation of PKC to the membrane, cause reorganization of the protein structure which probably causes the increased affinity for lipids. However, in order to investigate further the prevalence of the ligand-induced conformational changes in the soluble protein after binding to the phospholipids, we measured the c.d. spectrum in the lipid-bound form of PKC (Bazzi and Nelsestuen, 1991). Two methods were used to produce the lipid environment: PS vesicles and Triton X-100/PS mixed micelles, both in the presence of diacylglycerol. Only PS vesicles, however, turned out to be useful since micelles introduced high noise into the c.d. spectra. PKC incubated with PS/diacylglycerol vesicles lacking Ca^{2+} exhibited the c.d. spectrum shown as Figure 4, spectrum B, in which some reduction in the α -helix size was observed (Table 1). When Ca^{2+} was also present, a significant loss of α -helix structure was observed (Figure 4, spectrum C). The same results were obtained when PDBu was present in addition to the lipid vesicles (Figure 4, spectrum D), suggesting that the conformation of the soluble enzyme induced by Ca^{2+} and phorbols prevails after binding to phospholipids.

The finding by McFadden et al. (1989) that PKC may be autophosphorylated at low pH in the absence of Ca^{2+} and lipids prompted the study of the soluble enzyme at pH 4.5 in an attempt to differentiate between the changes elicited by Ca^{2+} and phorbols and those that strictly affect the catalytic site of the enzyme. Concerning PKC, autophosphorylation occurs in the

Figure 6 Fluorescence spectra of PKC in the presence of Ca^{2+} and phorbols

PKC (50 $\mu\text{g}/\text{ml}$) was excited at 295 nm and emission in the 290–400 nm range was recorded. (a) Spectrum at pH 7.5 of PKC in the absence of ligands (A) or in the presence of 100 μM Ca^{2+} (B), 100 μM Ca^{2+} plus 5 μM α -PDD (C) or 100 μM Ca^{2+} plus 2 μM PDBu (D). (b) Differential fluorescence (ΔF) between the spectrum of PKC at pH 4.5 and that at pH 7.5 assayed in the absence of ligands (A) or in the presence of 100 μM Ca^{2+} (B). Inset shows the ΔF of PKC recorded at 343 nm at several pH values. The pH changes were obtained by combining different amounts of 0.2 M acetate, pH 4.2, and 0.2 M Hepes, pH 7.5, to give a final buffer concentration of 40 mM.

course of expression of its catalytic activity and correlates with the exogenous phosphorylating capacity of the enzyme in the usual pH range (Huang et al., 1986). Hence this capacity may be used as a criterion to determine the degree of activation of the enzyme. Figure 5, spectrum B is the c.d. spectrum of PKC at pH 4.5 in the absence of activators (Ca^{2+} and PDBu) and lipids. The similarities were remarkable between this spectrum and that of the lipid-vesicle-associated enzyme obtained in the presence of either Ca^{2+} or PDBu (Figure 4). Again, a reduction in the α -helix size and content was observed.

Fluorescence analysis

The marked changes in the c.d. spectrum of PKC elicited by Ca^{2+} and PDBu prompted the study of whether this reorganization in the secondary structure of the protein correlated with changes in its intrinsic fluorescence. The enzyme contains six tryptophan residues which are the major contributors to the fluorescence of the protein (Parker et al., 1986). As Figure 6(a) shows, when PKC is excited at 295 nm, a peak of fluorescence is obtained at 340 nm. Incubation of PKC with 100 μM Ca^{2+} produced a 19% decrease in the intensity of fluorescence (maximal effect), without affecting the wavelength of emission. Addition of 5 μM α -PDD to the assay did not affect the emission maximum, although a 16% decrease was produced in the spectrum. However, when PKC was incubated in the presence of 100 μM Ca^{2+} and 5 μM PDBu, the spectrum was decreased and shifted to the red (peak maximum at 352 nm), suggesting that the effect produced by phorbol esters consisted of increasing the exposure of the fluorogenic groups to the polar medium. These results are in agreement with the specific responses produced by these PKC ligands on the c.d. spectra of the protein.

Furthermore, when the protein was incubated at pH 4.5 in the absence of activators, a decrease in the emission maximum of the fluorescence was observed (Figure 6b). However, this decrease in intensity was also enhanced by Ca^{2+} , suggesting that, with respect to the reorganization of the enzyme, the two conditions (pH and Ca^{2+}) were not alike. When the fluorescence was plotted at 340 nm as a function of pH, obtained by combining mixtures of solutions at pH 4.5 or 7.5, a clear pH-dependent curve was obtained. The profile was similar to that reported for the pH-dependent autophosphorylation of PKC in the absence of Ca^{2+} (McFadden et al., 1989).

DISCUSSION

In the present investigation, we considered the possible relationship between the changes in the secondary structure of the protein elicited after binding of PKC effectors and activation of the enzyme. In a recent study, a significant decrease in α -helix content of PKC was shown after binding of the enzyme to phospholipid vesicles (Shah and Shipley, 1992). However, our results deduced from the study of the effect of PKC ligands on both the far u.v. c.d. and the autofluorescence spectra of the protein suggest that these structural changes are induced after the synergistic binding of Ca^{2+} and phorbols to the protein.

The method employed to adjust the experimental and calculated c.d. curves (Menendez-Arias et al., 1988), by using the set of parameters proposed by Chang et al. (1978), provided excellent fits with respect to the different conditions analysed in our investigation. Alternatively, two other sets of proteins and peptides of known structure were used as reference models (Chen et al., 1974; Bolotina et al., 1980), although the fits between experimental and model curves were less precise than those obtained by Chang et al. (1978). This method allowed the calculation of the average number of amino acids in each α -helix segment. However, this calculation is only one of the possible ways to interpret the c.d. spectra on the basis of the characteristic displacement of the θ minima to the red. One objection to the use of c.d. analysis to deduce secondary structure of proteins is the great dependence of the structural parameters on the particular set of reference curves employed. However, the level of confidence in our analysis was reinforced by the good agreement observed between the structure deduced from the amino acid sequence of the protein (Parker et al., 1986) and that derived from the c.d. spectrum. This relationship should be interpreted with caution because the average secondary-structure prediction yields results that are correct only 60% of the time, regardless of the method used for the estimation (Kabasch and Saunderson, 1983). The reason for relatively low success rate of methods for structural prediction is unclear (Rooman and Wodak, 1988), but one possible explanation (Pace, 1990) is the low values of the conformational energy of globular proteins (the difference in free energy between the folded and unfolded state under physiological conditions).

Although quantitative determination of the secondary structure of proteins deduced by c.d. analysis has received criticism, we stress the comparison of the c.d. spectra of PKC in the presence of ligands with that obtained for the soluble protein. Indeed, the changes in c.d. may be interpreted in terms of differences between protein structure. This is especially relevant for the determination of α -helix content, since the extent of this structure may be accurately measured by c.d. at 220–222 nm. However, quantification of β -structure and β -turns requires c.d. measurement at 160–190 nm in media without absorption at this wavelength, a situation impossible to obtain under our instru-

mentation and experimental conditions (Johnson, 1988; Manalavan and Johnson, 1985; Toumadje et al., 1992).

Our results showed that binding of Ca^{2+} to PKC did not affect the structure of the protein. Similar results were obtained when PDBu was used as the ligand, which was due to the low affinity of the protein for phorbols in the absence of Ca^{2+} . Indeed, the synergism observed between Ca^{2+} and PDBu for the translocation of the enzyme was also evident in the structural changes in the protein elicited by both ligands. This synergism may also reflect some of the structural changes required to produce an active enzyme when, in addition to these activators, phospholipids are present. Moreover, the possibility that the conformation induced by Ca^{2+} and PDBu is due to the binding of PDBu to unspecific hydrophobic domains of the protein, in addition to the high-affinity binding site for the β -phorbols, or to the spectral properties of the phorbols, seems unlikely since the inactive phorbol ester α -PDD did not produce changes in either the c.d. spectrum or the fluorescence intensity of the tryptophan residues. Indeed, α -PDD was neither able to promote the translocation of PKC to lipid vesicles nor to activate PKC. Taken together, these results suggest that the structural changes produced by the synergistic action of Ca^{2+} and PDBu are related to their effects on the translocation and activation of the enzyme.

The data on the fluorescence of the tryptophan residues after binding of Ca^{2+} were in agreement with those reported for the three isoenzymes described in bovine brain (Huang, 1989). The shift to the red in the fluorescence peak intensity after binding of PDBu in the presence of Ca^{2+} again suggests the existence of a synergistic action between these two ligands that promotes a specific change in the protein structure, in agreement with their modifications of the c.d. spectrum. Moreover, these results agree with the suggestions that Ca^{2+} and PDBu favour the exposure of hydrophobic regions of the protein to the polar environment which may produce a decreased stability of the protein in solution and an increased affinity for phospholipids. Finally, the relevance of the shift in the fluorescence of PKC as an index of the activation state of the enzyme was reinforced by the observation of this shift when the protein was incubated at pH 4.5, a condition in which the enzyme exhibited its capacity to be autophosphorylated, although it lacked the ability to phosphorylate exogenous substrates (McFadden et al., 1989).

Finally, our results raise the question of why the binding of Ca^{2+} and PDBu to PKC produces such a significant reorganization in the α -helix segments. One possible answer is that α -helices, because of their properties in packing surfaces, may be organized around the hydrophobic core of globular proteins in structures in polyhedra-like shape (Murzin and Finkelstein, 1988). According to our results, since translocation of PKC was associated with a reduced average α -helix size and with exposure of hydrophobic domains to the polar medium, as reflected by the decrease in tryptophan fluorescence, we suggest that the reorganization in the α -helix structure is related to the accessibility of inner hydrophobic regions of the protein to lipid environments,

and therefore may explain the high efficiency of PKC translocation elicited by these ligands. Moreover, studies on the amino acid sequence required for this translocation from the cytosol to the membrane revealed that this motif, which contains basic amino acid residues, is quite conserved among several translocable enzymes (Clark et al., 1991).

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