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Inhibition of Substrate Binding to the Adrenal Cytochrome $P450_{C-21}$ by Acrylamide and Its Implications for Solvent Accessibility of the Binding Site in the Microsomes†

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ABSTRACT: The present study offers evidence indicating that acrylamide, a highly polar molecule and an efficient quencher of tryptophanyl fluorescence, inhibits substrate binding to $P450_{C-21}$ in bovine adrenocortical microsomes, in a competitive manner similar to that in the purified enzyme. Resolution of the fluoresence-quenching data revealed an acrylamide quenching constant (K_2 = 9.9 M, that is, the association constant for the quencher–fluorophore complex) that was similar to the reciprocal of its inhibition constant ($1/K_i = K_a = 8.3 \pm 0.9$ M) for substrate binding. The substrate inhibited the fluoresence quenching by acrylamide as indicated by its concentration-dependent decrease in K_2. The inhibition was in accordance with partial competition. These results are essentially similar to those previously observed in the purified lipid-free enzyme. In addition, the substrate dissociation, acrylamide inhibition, and fluorescence-quenching constants and the tryptophanyl fluorescence maximum (340–342 nm) were essentially the same in the microsomes and the lipid-free purified enzyme. These results indicate that the substrate-binding site of $P450_{C-21}$ and the concerned tryptophan are accessible to the highly polar molecule in the microsomal membranes, similar to that in the lipid-free purified enzyme. This implies that the substrate-binding site is not shielded by lipids in such a way that only the substrate in the lipid phase can gain access to the binding site. This conclusion is consistent with the currently favored model, for membrane topology of mammalian P450 enzymes, in which P450 is anchored to the membrane through a short N-terminal sequence while the remaining portion of the molecule is exposed to polar environment.

Cytochrome P450, the oxygen-activating component of a variety of mixed function oxidases, is ubiquitous in nature. Mammalian cytochrome P450 enzymes are mainly intrinsic membrane proteins, whereas bacterial P450's are water soluble. Binding of substrates to the soluble (Katagiri et al., 1968) as well as membrane-bound P450's (Narasimhulu, 1963, 1965; Remmer et al., 1966; Schenkman et al., 1967; Peterson et al., 1971) results in the characteristic blue-shift of the Soret absorption band. With this spectral shift as the criterion for binding, various aspects of substrate–P450 binding equilibria have been studied. Most substrates of P450 enzymes, including those of water-soluble ones, are largely hydrophobic. Studies using hepatic microsomal P450's have shown correlation of substrate hydrophobicity and substrate affinity (Al-Gailany et al., 1978; White et al., 1980), indicating that substrate-binding sites of P450 enzymes are probably hydrophobic. At present, most direct evidence regarding the nature of the binding site and its accessibility to substrate comes from a high-resolution X-ray crystallographic study of $P450_{cam}$ (Poulos, 1986). Studies on the crystal structure of substrate-bound $P450_{cam}$ have shown that the site, in which the substrate resides upon binding, is lined with hydrophobic residues. In addition, these studies have shown that camphor also makes polar contacts (with the hydroxyl group of Tyr-96). This suggests that polar interactions may also be involved in the substrate binding dynamics. Similar direct evidence is not yet available for the membrane P450's. However, there are significant similarities between membrane P450's and $P450_{cam}$. For example, sequence alignment studies have shown that all eukaryotic P450 species show clear homologies with the bacterial enzyme in the vicinity of the cysteine heme ligand (Cys-357 in $P450_{cam}$) and the oxygen-binding site (helix I), whereas other regions are less well conserved (Poulos et al.,

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1987; Goto et al., 1983; Nelson & Strobel, 1988), suggesting that all species share some common structural features while other parts of the structure differ due to different substrate specificity and due to the fact that the eukaryotic P450's are membrane proteins. In addition, there is reasonable evidence (Sagakuchi et al., 1984, 1987; De Lemos-Chiarandini et al., 1987; Nelson & Strobel, 1988; Brown & Black, 1989) indicating that the microsomal P450 is anchored to the membrane by a short amino-terminal loop, covering only 50–60 amino acids, while the remaining portion of the molecule is probably exposed to a polar environment. The exposed portion may be considered to be in an environment similar to that in the case of the water-soluble enzyme $P450_{cam}$. Therefore, the water-soluble bacterial enzyme may serve as a useful model system for eukaryotic P450's, although the latter are membrane bound.

The substrates of P450 enzymes being largely hydrophobic, they can also bind to or partition into the lipid phase of biological membranes. Therefore they can gain access to the binding site of P450 via the lipid phase provided the site is accessible from the lipid phase. Recently, Kominami et al. (1986) have studied the purified adrenal microsomal $P450_{C-21}$ in phosphatidylcholine (PC) vesicles. They measured substrate-binding and dissociation rates and considered the data in terms of the kinetic equations of Parry et al. (1976) for a site in the lipid phase (L model) and that in the aqueous phase (A model). Because (a) the steroid partitioning into lipid phase was more rapid than binding to P450, (b) the dissociation rate constant for the more lipid-soluble substrate was greater, and (c) in the L model the substrate is released into the lipid phase, Kominami et al. (1986) suggest that in PC vesicles the substrate-binding site is shielded by the lipid phase. This implies that only the substrate in the lipid phase can gain access to the binding site. The substrates of $P450_{C-21}$, progesterone, and 17-hydroxyprogesterone, although mainly lipid soluble, have sufficient water solubility in aqueous medium (Heap et al., 1970) when compared to their high affinities to the P450 ($K_d^{app} = 10^{-6}$–10^{-7} M).[1] In other words, the substrate-binding site on the enzyme would be saturated at a much lower substrate concentration than that at which the substrate begins to aggregate. Therefore, depending upon the binding site accessibility, these substrates may interact with the P450 in microsomes via the lipid or aqueous phase. Since there are no lipids in the soluble enzyme $P450_{cam}$, the binding site, although hydrophobic, must be accessible to camphor in the aqueous phase.

In the present study, solvent accessibility of the substrate-binding site in microsomes has been investigated by a different approach. Acrylamide, an uncharged polar molecule and efficient quencher of tryptophanyl fluorescence, has been frequently used to probe solvent accessibility of intrinsic fluorophores in proteins (Eftink & Ghiron, 1976, 1981; Calhoun et al., 1983). When a fluorophore in a protein is near or in the substrate-binding site, fluoresence offers an excellent spectroscopic technique to study the binding-site accessibility and location within the membrane. Previous studies (Narasimhulu, 1988) using the highly purified cytochrome $P450_{C-21}$ have shown that acrylamide inhibits the binding of 17α-hydroxyprogesterone to the cytochrome in a competitive manner, with an inhibition constant similar to the dissociation constant for the quencher–fluorophore complex. Therefore it was suggested that a tryptophan in a relatively polar environment is in the substrate-binding site or in its vicinity. Of greater physiological significance is the accessibility of the substrate-binding site in the more integrated system, the microsomes. The results of the present study indicate that the substrate-binding site in the microsomal membrane is accessible to acrylamide similar to that in the lipid-free enzyme, indicating that the site is not shielded by the lipid phase. This is unlike that reported for the P450 in PC vesicles (Kominami et al., 1986).

Materials and Methods

Materials

Acrylamide (4× crystallized) was a product of Serva Co., Heidelberg. Emulgen 913 was purchased from Kao Atlas Co., Tokyo. 17-Hydroxyprogesterone from Steraloid Co. was chromatographically (HPTLC) pure.

Methods

Preparation of Microsomes. Bovine adrenocortical microsomes were prepared essentially as previously described (Narasimhulu et al., 1985). A 20% homogenate of the cortex tissue in 0.3 M RNAase-free sucrose containing 1 mM EDTA and 0.005 M Hepes buffer, pH 7.4, was subjected to differential centrifugation as follows: The homogenate was centrifuged at 9000*g*, 10500*g*, and 12000*g* for 15 min at each speed, and the sediment at the end of each run was discarded. The 12000*g* run was repeated until the amount of sediment formed was insignificant. The 12000*g* supernatant was centrifuged at 78000*g* for 1 h. The sedimented microsomes were washed with 0.15 M KCl. The washed microsomes were suspended in 0.3 M RNAase-free sucrose containing 0.005 M Hepes buffer, pH 7.4, to a protein concentration of about 20 mg/mL and stored at −70 °C until use. When the 17-hydroxyprogesterone-induced type I spectral change (ϵ mM^{-1} cm^{-1} 407–421 nm = 64.5) could account for significantly less than 75–80% of the total P450 (ϵ mM^{-1} cm^{-1} 450–490 nm = 91), the presence of endogenous steroids was assumed and the microsomes were treated with an NADPH-generating system. After such treatment, the spectral change induced by exogenous steroid could account for 75–80% of the total P450 present in the microsomes. The total P450 per milligram of protein was essentially unaltered by this treatment. The total P450 content of different batches of microsome preparations has varied from 0.47 to 0.55 nmol per milligram of protein determined by the usual Biuret procedure as described by Gornal et al. (1949).

Purification of $P450_{C-21}$. The procedures for purification of the P450 by aminooctylamine-Sepharose (AOA-Sepharose) column chromatography, removal of the detergent (Emulgen 913) used in the purification procedure, and estimation of the residual detergent was as previously described (Narasimhulu et al., 1985). The detergent concentration of the concentrated (2.6 μM) P450 was 0.01%. On this basis the detergent concentration in the experiments reported was about 60-fold diluted, which was considered negligible.

Lipid Determination in Purified P450. The procedure was essentially as previously described (Marmer & Maxwell, 1981). A total of 5 nmol of purified P450 in 200 μL were subjected to Folch-type extraction with chloroform and

[1] Abbreviations: K_a, equilibrium association constant; K_i, inhibition constant; K_1 and K_2, quenching constants for the two fractions of the resolved fluoresence (frac I and frac II); K_d^{app}, apparent dissociation constant; K_p, partition constant (lipid/aqueous); V_{lip}, volume of the lipid phase; V_T, total volume; E_T, total enzyme; χ^2 = chi square; ϵ, dielectric constant.

methanol. The entire chloroform layer was applied to Whatman HPTLC plate (Silica gel 5 μm) and developed with chloroform/methanol/water (65:25:4). Visualization was accomplished by (a) UV absorption after spraying with 1,6-diphenylhexatriene, (b) after Phospray, and (c) after charring. There were no detectable phospholipids. By this procedure, a minimum of about 100 ng of the lipid can be detected.

Determination of the Apparent Substrate Dissociation Constant (K_d^{app}). Absorption spectra were obtained with a split beam Perkin-Elmer spectrophotometer model 577. The type I difference spectrum of cytochrome P450 induced by substrate binding, which is characterized by a minimum at 421 nm, a broad maximum at 388–390 nm, and an isosbestic point at 407 nm, was used as the criterion for the binding of the substrate to the enzyme. The procedure for spectral titration with the substrate by a semimicrotitration technique was as previously described (Narasimhulu, 1979). A cuvette of 12-mm light path containing 3.0 mL of the assay system was placed in a dual wavelength filter photometer fitted with interference filters of 421 (λ_1) and 407 nm (λ_2) of 1-nm half-bandwidth. The assay system was constantly stirred during the titration with a magnetic stirring attachment. The temperature was regulated by a thermostated circulator and measured with a thermocouple. An appropriate concentration of methanolic solution of the steroid was added in 0.1-μL aliquots by using a 10-μL gas-tight syringe attached to a programmable Hamilton precision dispenser. After each addition, the absorbance difference ΔA (407–421 nm) was recorded with a strip chart recorder. The assay system consisted of 33 mM potassium phosphate buffer, pH 7.25, containing 7% glycerol, 0.2 M KCl, 0.14 M RNase-free sucrose, and the desired concentrations of microsomes or purified enzyme preparation.

Stopped Flow Measurements. The kinetics of substrate binding to $P450_{C\text{-}21}$ were measured with a Hi-Tech stopped-flow apparatus (Hi-Tech, Ltd., Salisburg, U.K.). Five volumes of microsomes suspended in 0.27 M sucrose containing 0.01 M glycylglycine buffer, pH 7.25 ($[P450_{C\text{-}21}] = 0.3$ μM), were mixed at 22 °C with one volume of the same buffer containing methanolic solution (4 μL methanol/mL) of 17α-hydroxyprogesterone. The resulting absorbance changes were monitored at 420 or 390 nm, with a 2-mm path length. Binding rates were determined by nonlinear least-squares analysis of the single-exponential relaxation data obtained in the presence of excess substrate.

Determination of the Fluorescence-Quenching Constant for Acrylamide. All steady-state fluorescence measurements were made with a Perkin-Elmer model 650-10S fluorescence spectrometer. The excitation wavelength was 295 nm. A 1-mL aliquot of the assay system having an optical density no greater than 0.05 at 295 nm was used. The temperature of the assay system was maintained at 26 °C. The tryptophanyl fluorescence was monitored at 340 nm. The details of the procedure, small corrections for sample dilution, and attenuation of the excitation light intensity by the added acrylamide were applied as previously described (Narasimhulu, 1988).

Fluoroscence Lifetime Measurements. Lifetime measurements were made with a time-correlated single photon counting instrument, as previously described (Narasimhulu, 1988), except that instead of a pumped argon laser the second harmonic of a CW-Nd-YAG laser was used (Coherent, Model Antares).

Data Analysis

Fluorescence Data. The steady-state fluorescence-quenching data were analyzed according to the different forms (see eqs 4–7) of the well-known Stern–Volmer equation. The program used for analysis was a curve-fitting program based on the algorithm of Marquardt (1963). The standard error of estimate, randomness of distribution of residuals, and reduced χ^2 were considered in evaluating the goodness of fit. In order to correct for systematic errors, we performed five identical titrations covering the quencher concentration range used in actual experiments and constructed a standard curve of standard deviation versus quencher concentration. This allowed estimation of the σ value for each data point in steady-state quenching experiments. These σ values were used in the curve-fitting procedure.

Substrate-Binding Data. Substrate-binding data were analyzed as previously described (Narasimhulu et al., 1985), according to eq 1, by using a nonlinear least-squares fitting algorithm (Marquardt, 1963).

$$[S]_f/[ES] = K_d/[E_t] + [S]_f/[E_t] \quad (1)$$

where $[S]_f$ is the free substrate concentration and [ES], the concentration of the enzyme–substrate complex, and $[E_t]$, the concentration of the total enzyme, are proportional to ΔA and ΔA_{max}, respectively. They were calculated by using an extinction coefficient for the substrate-induced type I difference spectrum of (76.8 mM^{-1})(1.2 cm^{-1}) for the wavelength pair of 407–421 nm (Narasimhulu et al., 1985). The 95% confidence interval for K_d^{app} was determined from the standard deviations for the slope and the intercept according to the procedure of Bevington (1969). Similarly, by use of the standard deviation for K_d^{app}, the confidence interval for the acrylamide inhibition constant (K_i) was calculated.

Results

The Effect of Microsome Concentration on the Apparent Equilibrium Dissociation Constant for the 17α-Hydroxyprogesterone-$P450_{C\text{-}21}$ Binding Reaction. Any membrane suspension may be regarded as a biphasic system, the phases being the lipid bilayer of the membrane and the aqueous suspending medium. Lipid-soluble molecules such as substrates of cytochrome P450 enzymes, when added to a membrane suspension, can partition into the lipid phase. Therefore, in the case of a membrane-bound enzyme, the partitioning of a lipid-soluble substrate into the lipid phase may mask the true value for the apparent dissociation constant for the enzyme–substrate complex (Parry et al., 1976). Kominami et al. (1986) have found that, at constant $P450_{C\text{-}21}$ concentration, K_d^{app} increased with increasing PC concentration. In addition, in hepatic microsomes, K_d^{app} for androstenedione increased with an increase in membrane concentration (Ebel et al., 1978). In the present study, it was necessary to use different microsome concentrations for optimal operation in determining substrate-binding and fluorescence-quenching parameters because of considerable differences in the sensitivities of the two techniques. Therefore it was important to test the effect of microsome concentration. Since higher concentrations were needed for substrate-binding studies than fluorescence studies, the effect of microsome concentration on the apparent K_d of 17α-hydroxyprogesterone (the substrate used in the present study) was tested. This was important especially because the partition constant for this steroid in lipids is high. For example, in phosphatidylcholine vesicles, it is 2000 in favor of the lipid phase (Kominami et al., 1986).

The results of experiments on the effect of microsome concentration on the K_d, performed at 25.1 and 37.9 °C, are shown in Table I. Within the protein concentration range

Table I: Effect of Microsome Concentration on the Substrate–$P450_{C\text{-}21}$ Binding Parameters[a]

protein (mg/mL)	K_d (μM)		$[ES]_{max}$ (μM)	
	25.1 °C	37.9 °C	25.1 °C	37.9 °C
0.08	0.085	0.219	0.03	0.028
0.13	0.087	0.204	0.045	0.046
0.25	0.08	0.208	0.094	0.092

[a] Standard error of estimate for the overall analysis ranged from 0.8 to 2.9% of median [ES] values.

studied (0.08 to 0.25 mg of protein/mL), the maximum type I spectral change increased linearly with an increase in microsome concentration. As previously reported (Narasimhulu, 1979), the apparent K_d increased with an increase in temperature. However, it was independent of the microsome concentration even at the higher temperature at which the solubility of the steroid in the lipid phase would be expected to be greater. Thus, in the microsomes used in the present study, any partitioning of the steroid into the lipid phase was without effect on the K_d, indicating that the total substrate present is accessible to the P450 at equilibrium. In otherwords $K_d^{app} = K_d$, which is possible only if either the microsome concentration or K_p (partition constant, lipid/aqueous) is sufficiently low, as indicated by the following equation for a site accessible to the aqueous phase (Parry et al., 1976).

$$K_d^{app} = K_d[K_p k(E_t) + 1]$$

$$V_{lip}/V_T = k(E_t)$$

where V = volume and V_T = total volume. The K_d^{app} was calculated according to this equation, taking into account the amount of the added substrate in the lipid phase. The substrate in the lipid phase was calculated according to Parry et al. (1976), assuming $K_p = 2000$, the ratio of lipid to protein = 0.4 by weight (De Pierre & Ernster, 1977), and the density of lipid = 0.9 g/mL (Comte et al., 1976), and $K_d = 0.08$ μM (Table I). These calculations indicated that an increase in the microsome protein concentration from 0.08 mg/mL ($V_{lip} = 3.6 \times 10^{-5}$ mL) to 0.25 mg/mL ($V_{lip} = 1.2 \times 10^{-4}$ mL) should have increased the K_d^{app} from 0.086 to 0.098 μM. This is an increase of 14%, which is greater than twice the standard deviation (3.3) for determining the apparent K_d in microsomes, calculated from the results of five titrations under identical conditions (Narasimhulu et al., 1985). Therefore the 14% increase should have been detectable under the present experimental conditions. Since the measured values of K_d^{app} were found to be independent of protein concentration, the value of K_p of 17α-hydroxyprogesterone for microsomal lipids must be less than 2000. Therefore the value of K_p worked out for pure liposomes in the absence of proteins is probably not applicable to microsomes. The equation for the A model was selected because the results of the present study indicated that the substrate-binding site of the P450 in microsomes is probably accessible to the aqueous phase similar to that in the purified lipid-free enzyme.

Lipid Content of Purified $P450_{C\text{-}21}$. If sufficient amounts of residual lipids are present in the purified enzyme, it may be possible for the substrate-binding site to be shielded by lipids. In that case, comparison of the purified enzyme with the membrane-bound enzyme may not reveal any differences between them. Therefore, attempts were made to detect any residual lipids in the purified enzyme. Lipids could not be detected by HPTLC. If lipids are present, it must be less than 0.1 mol/mol of P450. It is unlikely that this amount of lipid is sufficient to mask any differences between the purified enzyme and the microsomes. As indicated under Methods, the residual detergent concentration may be considered negligible under the experimental conditions.

Table II: Substrate–$P450_{C\text{-}21}$ Binding Parameters Competitive Inhibition by Acrylamide

prep	[acryl] (M)	K_d^{app} (μM)	$[ES]_{ma}$ (μM)	$K_a = 1/K_i$ (M^{-1})	SEE[a] (% median [ES])
microsomes	0	0.11	0.08		0.61
	0.12	0.23	0.083	8.3 ± 0.7	1.2
	0.23	0.38	0.079	9.9 ± 0.9	2.5
purified P450	0	0.17	0.043		0.9
	0.235	0.51	0.039	8.3 ± 0.8	0.8

[a] SEE is the standard error of estimate for the entire curve.

Inhibition of Substrate–P450 Binding by Acrylamide. As in the case of the purified lipid-free $P450_{C\text{-}21}$ (Narasimhulu, 1988), the Lineweaver–Burk plots of the substrate-binding data obtained by titrating the type I spectral change with the substrate 17α-hydroxyprogesterone were linear, both in the absence and in the presence of acrylamide. Acrylamide (0.23 M) increased the apparent substrate dissociation constant from 0.11 to 0.38 μM (Table II) without a significant effect on the maximum concentration of enzyme–substrate complex formed. The degree of inhibition depended upon the inhibitor to substrate ratio. Previous studies have shown that the inhibitor does not bind to the substrate and the substrate does not alter the position of the tryptophan emission maximum or the intensity in the purified enzyme (Narasimhulu, 1988), ruling out other mechanisms that may result in apparent competition (Dixon & Webb, 1979). Essentially similar results were obtained with the microsomes, in the present study. Therefore the binding data were analyzed as previously described according to the equation for competitive inhibition.

$$1[ES] = 1/[ES]_{max} + (1/[ES]_{max})(K_d + K_d[i]/K_i)1/[S] \quad (2)$$

K_d is the apparent dissociation constant of the substrate and K_i is the inhibition constant that is actually the dissociation constant of the inhibitor. The K_i was calculated from the following equation for the "X" intercept:

$$K_i/(K_d K_i + K_d[i]) = 1/[S]_1 \quad (3)$$

The range of values obtained for K_i at two different concentrations (0.12 and 0.23 M) of the inhibitor were essentially identical (0.11 and 0.12 M). This gives an equilibrium association constant for acrylamide of 8.3–9.9 M. These results indicate that although the affinity of acrylamide to the enzyme is six orders of magnitude lower than that of the substrate, the inhibitor competes with the substrate, at sufficiently high concentrations. This is consistent with the classical equation for competitive inhibition, which indicates that competition depends upon the ratio $[i]/K_i$ and not on the relative affinities of the substrate and the inhibitor. This equation is applicable to the present case because the high inhibitor concentrations required to compete with the substrate do not cause any irreversible damage to the enzyme (Narasimhulu, 1988). The acrylamide inhibition constant obtained in the present study with the microsomes is similar to that in the case of the purified $P450_{C\text{-}21}$ (Table II).

Kinetics of Substrate Binding to $P450_{C\text{-}21}$ in Microsomes. Competition between the substrate and acrylamide in the substrate-binding reaction in the microsomes, which is similar to that in the purified lipid-free P450, is consistent with the accessibility of the binding site of the membrane-bound enzyme to the aqueous medium. In the PC-reconstituted enzyme, the

Table III: Rate Constant for the Substrate–$P450_{C-21}$ Association Reaction in Microsomes

17-hydroxy-progesterone (μM)	k_{obs} (s^{-1})	k_{ass} (mol^{-1} s^{-1})
0.65	6	9.2×10^6
2.2	23	10×10^6
4.3	40	9.3×10^6

second-order rate constant observed by Kominami et al. (1986) (7×10^6 M^{-1} s^{-1}), estimated from their Figure 5, is within the same order of magnitude as that (10×10^6 M^{-1} s^{-1}) in their A model and three orders of magnitude higher than in their L model. Therefore, it was of interest to find out whether or not the rate constant for substrate binding in microsomes is similar to that observed by Kominami et al. (1986) for the PC-reconstituted enzyme. The rate of substrate–P450 complex formation was measured by using the type I spectral change as the criterion for binding. Either the decrease in absorbance at 420 nm or the increase at 390 nm, induced by substrate binding, was monitored as a function of time by the stopped flow technique. The observed pseudo-first-order rate constant depended linearly on substrate concentration, indicating that the rate-limiting step of the substrate-induced spectral change is the bimolecular substrate-binding reaction. The average second-order association rate constant was 9.5×10^6 M^{-1} s^{-1} (average of the values in Table III), which is very similar to that observed by Kominami et al. (1986) for the PC-reconstituted enzyme. If the steroid partitioning into lipid phase is more rapid than binding to P450, similar to that in the PC-reconstituted enzyme, it is possible that the second-order rate constant is underestimated. Assuming that the steroid partitioning into lipid is more rapid than binding to P450 (Kominami et al., 1986) and a K_p of 2000, 25% of the total steroid was calculated to be in the lipid phase. If this is the case, the second-order rate constant (Table III) would increase from 1×10^7 to 1.6×10^7 M^{-1} s^{-1} (at 2.2 μM 17α-hydroxyprogesterone), but still consistent with the A model. In the microsome preparation used in the stopped flow experiments, the dissociation constant determined from equilibrium binding studies was 0.1×10^{-6} M. When this value and the second-order rate constant shown in Table III were used, a dissociation rate constant of 0.95 s^{-1} was calculated. The dissociation as well as the binding rate constants appear to be sensitive to solvent conditions. For example, the equilibrium and kinetics of substrate binding were affected by addition of a nonionic detergent to the microsomal suspensions. In the presence of 0.3% Emulgen 913, the second-order rate constant (7×10^5 M^{-1} s^{-1}) was an order of magnitude lower, the K_d^{app} (0.6×10^{-6} M) was 6-fold higher, and the calculated dissociation rate constant (0.4 s^{-1}) was 2-fold lower than in the absence of the detergent.

Tryptophanyl Fluorescence Quenching by Acrylamide in Microsomes: Fluorescence Spectra of Microsomes. Excitation of microsomes at 295 nm exhibits a broad fluorescence emission maximum at 340–342 nm (Figure 1). The fluorescence quenching by acrylamide was monitored at 340 nm. The long wavelength excitation minimizes contribution by tyrosine fluorescence as well as intertryptophanyl energy transfer (Teale, 1960). Therefore, total fluorescence may be assumed to be a sum of contributions by the individual tryptophans. The tryptophanyl residues in native proteins have been classified (Teale, 1960) into three distinct spectral classes: (a) buried in nonpolar regions (λ_{max} of 330–332 nm); (b) on the surface that is completely exposed to water (λ_{max} = 350–353 nm); and (c) in limited contact with water (λ_{max} = 340–342 nm). The 340-nm emission peak observed in microsomes suggests that the tryptophans are in limited contact with water, in an environment with a dielectric constant (ϵ) similar to that of methanol (ϵ = 32.6) or propanol (ϵ = 20.1; Cowgill, 1967). But the presence of tryptophans in both polar and nonpolar environments could have a composite emission peak at 340 nm. However, most of the fluorescence was accessible for quenching by the polar molecule acrylamide, as indicated by a value nearly equal to 1 for the "*Y*" intercept of the Lehrer plot (eq 8). In addition, there were no significant observable shifts in the position of the broad emission peak at the quencher concentrations tested (Figure 1). Considering that $P450_{C-21}$ alone contains 12 tryptophan residues (Yoshioka et al., 1986; Chung et al., 1986), it is very possible that there may be shifts (2–5 nm) in the broad peak position that are not detectable under the present experimental conditions and/or the tryptophans may be in similar environments or their fluorescence may be quenched. In P450 enzymes, the tryptophanyl fluorescence is probably largely quenched by the heme as indicated by a striking increase in the low fluorescence of a P450 enzyme when the heme was removed (Inouye & Coon, 1985), which is similar to that in other heme proteins (Teale & Webber, 1959). The fluorescence of $P450_{C-21}$ is also rather low (Narasimhulu, 1988). It should be pointed out that $P450LM_2$ exhibits a peak at 335 nm upon excitation at 295 nm (Inouye & Coon, 1985), indicating that the tryptophanyl environment in this P450 is probably more hydrophobic (ϵ = 15) than in $P450_{C-21}$.

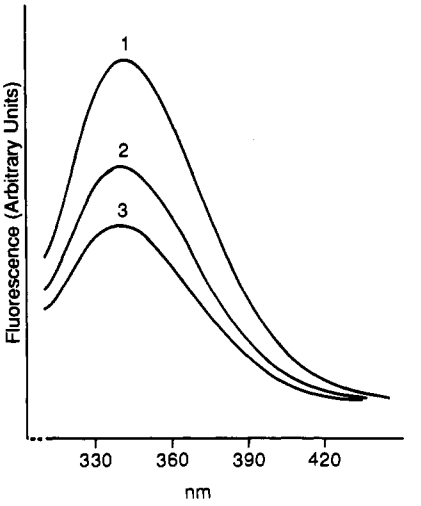

FIGURE 1: Fluorescence spectra of microsomes (approximately 0.1 mg of protein/mL) in the absence (curve 1) and in the presence (curves 2–3) of 0.167 and 0.335 M acrylamide. Microsomes were suspended in a buffer mixture consisting of 50 mM potassium phosphate buffer, pH 7.25, 10% glycerol, and 0.18 M KCl. The temperature was 26 °C, and the excitation wavelength was 295 nm.

Resolution of Tryptophanyl Fluorescence of Microsomes. (a) In the case of the purified $P450_{C-21}$ (Narasimhulu, 1988), the fluorescence-quenching constant of acrylamide was similar to the reciprocal of its inhibition constant for substrate binding; (b) in the purified enzyme, the substrate inhibited the fluorescence quenching by acrylamide; and (c) acrylamide inhibited substrate binding competitively in microsomes also, with a K_i similar to that in the purified enzyme. Therefore, it was of interest to find out if a quenching parameter similar to $1/K_i$ could also be demonstrated in the microsomes and if so whether or not this parameter is sensitive to substrate.

Steady-State Fluorescence-Quenching Experiments

Selection of the Best Fitting Model To Describe the Quenching Data. It is assumed that the observed tryptophanyl fluorescence is a sum of contributions by two different types

Table IV: Comparison of the Different Models for Fluorescence Quenching by Acrylamide

eq no.	f_1	K_1 (M^{-1})	f_2	K_2 (M^{-1})	SEE[a] (% med fl)	χ^2
4	0.62	3.7	0.38	4.7	3.8	268
5	0.76	3.3	0.24	6.5	1.0	4.1
6	0.68	2.4	0.32	9.9	0.4	1.2
7	0.86	2.5	0.14	17.3	0.3	1.6

[a] SEE, the standard error of estimate for the entire curve is expressed as percent median fluorescence.

Table V: Parameters of Tryptophanyl Fluorescence Quenching by Acrylamide in Microsomes (The Effect of 17α-Hydroxyprogesterone)[a]

17α-hydroxyprogesterone (μM)	f_1	K_1 (M^{-1})	f_2	K_2 (M^{-1})	SEE (% med fl)	χ^2
0	0.7	2.4	0.3	9.9	0.4	1.2
0.32	0.5	2.1	0.5	6.9	0.3	1.3
1.30	0.4	2.3	0.6	4.3	0.5	1.1

[a] The parameters were determined as described under Methods by fitting the data to Stern–Volmer eq 6.

of interactions of the quencher and the fluorophore, according to the following forms of Stern–Volmer equations:

$$F/F_0 = f_1/(1 + K_1[Q]) + f_2/(1 + K_2[Q]) \quad (4)$$

$$F/F_0 = f_1/(1 + K_1[Q]) + f_2/\exp K_2[Q] \quad (5)$$

$$F/F_0 = f_1/\exp K_1[Q] + f_2/(1 + K_2[Q]) \quad (6)$$

$$F/F_0 = f_1/\exp K_1[Q] + f_2/\exp K_2[Q] \quad (7)$$

where f_1 and f_2 ($f_1 + f_2 = 1$) are the two fractions and K_1 and K_2 are the quenching constants. Since f_1 and f_2 are not interchangable for given values of K_1 and K_2, eqs 5 and 6 are not symmetrical, and therefore they represent different models. The quenching data were analyzed (as described under Methods) by fitting the data to each one of the above equations and comparing the goodness of fit.

Acrylamide concentrations used in these studies were lower than 0.29 M because, when higher concentrations were tested in the case of the purified enzyme, the inhibition of the substrate–P450 binding reaction was not readily reversible (Narasimhulu, 1988). The different models are compared in Table IV. As indicated under Methods, the best model was chosen based on the standard error of estimate (SEE), χ^2 values, and randomness of distribution of the residuals. Equations 4 and 5 failed to fit the data, as indicated by the high values for SEE and χ^2 (Table IV). The χ^2 and SEE for eq 6 was not significantly different from those of eq 7, on the basis of the F test [variance ratio test (Bevington, 1969)]. However, the K_2 value of 17.3 M obtained in model 7 was considerably higher than in model 6 and inconsistent with all other results. The value of 9.9 M in model 6 was within the range of values for the acrylamide association constant ($1/K_i$ = 8.7–11.6 M^{-1}) determined from its inhibition constant (K_i) for substrate binding. In addition, the quenching constant as well as $1/K_i$ for the purified P450 are also within the same range (Narasimhulu, 1988). Thus, the values of parameters for fluorescence quenching according to model 6 are consistent with all other parameters observed for the interaction of substrate and acrylamide in the substrate-binding and fluorescence-quenching reactions. Therefore, model 6 was considered to be the more appropriate model to describe the data.

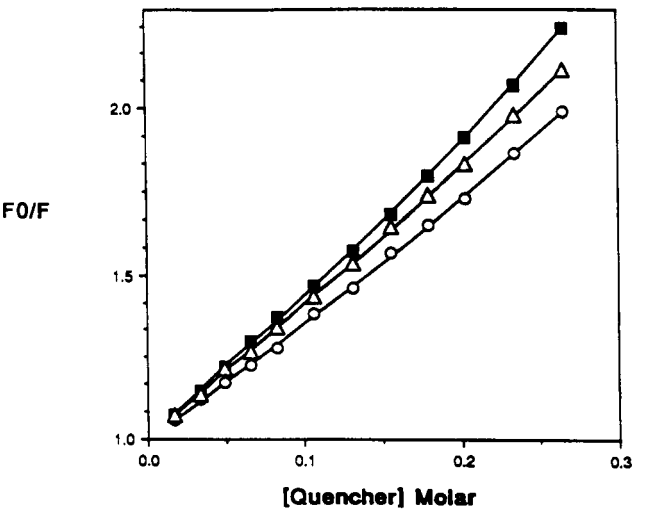

FIGURE 2: Stern–Volmer plots of fluorescence quenching by acrylamide. The assay system was as in the legend of Figure 1. Excitation and emission wavelengths were 295 and 340 nm, respectively. The temperature was 26 °C. The figure shows the goodness of fit of the theoretical curves (solid lines) to the experimental data in the absence (solid squares) and in the presence of 0.32 μM (triangles) and 1.3 μM (open circles) of 17α-hydroxyprogesterone. The theoretical curves are for eq 6 (Table V).

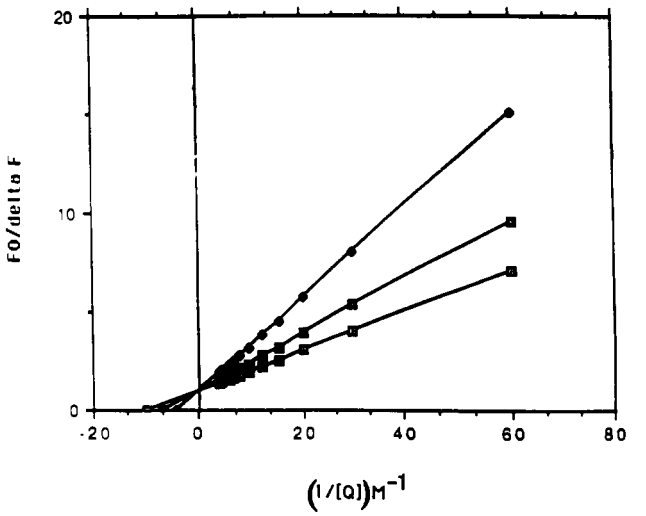

FIGURE 3: Lehrer plots of resolved fluorescence in the absence (curve 1) and in the presence of 0.32 μM (curve 2) and 1.3 μM (curve 3) of 17α-hydroxyprogesterone. These are theoretical curves for the second term, containing f_2 and K_2, of eq 6. The F_0 in the figure was the limiting value approached by the second term as the quencher concentration approached zero.

Figure 2 shows the Stern–Volmer plots of the steady-state quenching data for total fluorescence, in the absence and presence of the substrate 17α-hydroxyprogesterone. Within the quencher concentration range studied (0.017–0.26 M), a small curvature was apparent in the Stern–Volmer plot. A possible reason for the curvature is that the plot represents two types (static and dynamic) of quenching (Eftink & Ghiron, 1976, 1981). The results of resolving the observed fluorescence into two fractions according to eq 6 are shown in Table V. The substrate considerably decreased K_2 (the quenching constant for the second term of eq 6) but had little or no effect on K_1 (the quenching constant for the first term of eq 6). According to the present data analysis, the decrease in the first term caused by the substrate results in a corresponding increase in the second term. This is reasonable because the substrate was essentially without effect on total fluorescence and total

quenched. The decrease in K_2 depended on the concentration of the steroid (Table V), and the reciprocal plots according to Lehrer (Figure 3) were linear. The competition between acrylamide and the substrate in the substrate-binding reaction and correlation of the inhibition constant with the fluorescence-quenching constant suggested that the reaction of acrylamide with the fluorophore is a mass action equilibrium binding as previously reported (Narasimhulu, 1988). In addition, fluorescence lifetime data indicated that K_2 probably represents a static quenching constant, that is, an acrylamide association constant. Therefore, fluorescence quenching according to the second term of eq 6 can be considered in terms of binding equilibria. Then the ΔF, the quenched fluorescence, represents the quencher–fluorophore complex [Trp·Q], and F_0 represents the total fluorescence, which in the present case represents quenchable fluorescence $[Trp{\cdot}Q]_{max}$. Then according to the classical equation for competitive inhibition for equilibrium binding (Dixon & Webb, 1979)

$$\Delta F = F_0 - F$$

$$[Trp{\cdot}Q]_{max}/[Trp{\cdot}Q] = F_0/\Delta F = 1 + [K_d + K_d[i]/K_i]1/[Q] \quad (8)$$

Thus a plot of $F_0/\Delta F$ versus $1/[Q]$ (Lehrer plot) gives a straight line with a "Y" intercept = 1. The "X" intercept would have the same meaning as in the usual Lineweaver–Burke plot. Therefore, the reciprocal plots with a common "Y" intercept would indicate that the substrate competes with acrylamide in the fluorescence-quenching reaction. But the steroid inhibition constant (K_i = 0.7 μM) was considerably higher than its apparent dissociation constant (0.11 μM) determined from the substrate-binding data (Table II). This discrepancy may be due to the fact that acrylamide can interact with tryptophanyl groups in more than one way and quench the fluorescence (Eftink & Ghiron, 1981). Therefore, acrylamide may interact with the enzyme–substrate complex and quench the fluorescence, partially masking the inhibition by the substrate. Under such circumstances, the inhibition of acrylamide fluorescence quenching by the steroid is analogous to partially competitive inhibition (Dixon & Webb, 1979). This, however, is not in contradiction with the expected competition between the steroid and acrylamide in the quenching reaction. An alternative explanation for the lack of correlation is that f_2 fluorescence (Table IV, the second term in eq 6) represents a composite fluorescence of more than one tryptophan with similar acrylamide quenching constants and that the substrate interacts with one tryptophan. The discrepancy between the steroid dissociation constant and inhibition constant is similar to that observed in the case of the purified enzyme (Narasimhulu, 1988), indicating that the higher value for the inhibition constant is probably not due to nonspecific binding of the steroid to other structures in the microsomes.

Fluorescence Lifetime Measurements. Steady-state fluorescence-quenching data cannot distinguish between quenching by ground-state complex formation (static quenching) and collisional quenching. In the present study, it was important to find out if K_2 represents static quenching or collisional quenching. Since static quenching would have no effect on lifetimes, whereas collisional quenching would, fluorescence decay measurements were made in the presence of increasing concentrations of the quencher. The decay curves were best described by a sum of three exponential functions (χ^2 ranging from 1.1 to 1.2). In the absence of the quencher, the three lifetimes (from two measurements) were 2.03, 5.5, and 0.48 ns, respectively. In order to compare the quenching parameters obtained from lifetime data and those determined from steady-state fluorescence-quenching data, it was important to determine the quenching constant for the total fluorescence involved in collisional quenching. This was accomplished by adding the contributions of fluorescence represented by the three lifetimes, obtained by multiplying lifetimes and their respective amplitudes. The data were then analyzed according to the Stern–Volmer equation ($F_0/F = 1 + K_{sv}[Q]$). The collisional quenching constant (K_{sv}) determined by weighted linear regression analysis was 2.92 ± 0.6 M. The substrate 17α-hydroxyprogesterone was without effect on K_{sv}. This value for K_{sv} is similar to that of K_1 obtained from the steady-state quenching data (Table V).

Discussion

The present study offers evidence indicating that the substrate-binding site of $P450_{C\text{-}21}$ in the microsomes is accessible to the polar molecule acrylamide, similar to that in the purified lipid-free enzyme (Narasimhulu, 1988). This implies that the binding site in the membrane-bound P450 is accessible to (the substrate in) the aqueous medium.

Competition between the substrate and acrylamide in the substrate–P450 binding reaction indicates that a polar site which is not shielded by hydrophobic regions is involved in binding. Since competition occurs with similar acrylamide inhibition constants (K_i = 0.1–0.13 M) in the microsomes and the purified enzyme, the concerned polar site must be accessible to the substrate and the inhibitor regardless of whether or not the lipids are present. This implies that the substrate-binding site of P450 in the microsomes is probably not shielded by the hydrophobic regions of either the enzyme or the bilayer in such a way that the substrate must gain access only via such regions. These observations are consistent with the accessibility of the binding site to aqueous solvent in the presence of lipids.

Similarity of the tryptophanyl emission peak position in the microsomes (340 nm) and the purified enzyme (342 nm) indicates the presence of tryptophans in the membrane in an environment similar to that in the purified enzyme (Narasimhulu, 1988). As indicated under Results, the 340-nm peak suggests that the fluorophore environment is relatively polar (Cowgill, 1967). This is in accordance with the accessibility of the observed fluorescence for quenching by the polar molecule. As also indicated earlier, on the basis of the similarity of the acrylamide fluorescence-quenching constant and reciprocal of its inhibition constant ($1/K_i$) for substrate binding in the highly purified enzyme, it was suggested that a tryptophan is present either in the substrate-binding site or in its vicinity (Narasimhulu, 1988). If this is the case, there must be a quenching constant similar to $1/K_i$ in microsomes also. Resolution of the fluorescence of microsomes into two fractions, according to eq 6, resulted in f_1 with the quenching constant (K_1 = 2.4 M^{-1}) similar to that for collisional quenching (K_{sv} = 2.9 M^{-1}) determined from fluorescence lifetime data. This indicates that the first term of eq 6 represents collisional quenching, since static quenching would have no effect on fluorescence lifetimes. Then the higher value for the acrylamide quenching constant (K_2 = 9.9 M^{-1}) observed for f_2 must represent quenching by complex (acrylamide–tryptophan) formation. The similarity of the values for K_2 (the association constant for acrylamide) and the reciprocal of acrylamide inhibition constant ($1/K_i$, Tables II and V) for substrate binding suggests that the acrylamide binding site for fluorescence quenching and inhibiting substrate binding is one and the same. If this is the case, the substrate must inhibit acrylamide quenching of f_2 fluorescence, in a competitive manner. In fact, the substrate (17α-hydroxyprogesterone) does

inhibit acrylamide quenching of f_2 fluorescence, as indicated by the decrease in K_2 (from 9.9 to 4.2 M^{-1}). The decrease of K_2 in the presence of the substrate, while the "Y" intercepts of the reciprocal plots remained the same, is consistent with competition between the substrate and acrylamide in the quenching reaction. The lack of effect of substrate on K_1 or K_{sv} is consistent with K_1 representing collisional quenching and suggests that the quenching, according to the first term of eq 6, is entirely nonspecific. If there is contribution by nonspecific (static) quenching to f_2, the value of the quenching constant (K_2) must be the same as that of specific quenching. This is because the reciprocal plots of the resolved f_2 fluorescence were linear. Therefore, any contribution from nonspecific fluorescence to f_2 would not be expected to interfere with the present conclusion that the substrate and acrylamide compete for the same binding site in the quenching reaction.

Considering that the substrate binding rate constant was sensitive to solvent conditions, the similarity of the rate constant in microsomes [9.5×10^6 M^{-1} s^{-1} (average of values in Table III)] and that observed by Kominami et al. (1986) in PC vesicles, as well as in their A model (10×10^6 M^{-1} s^{-1}), is consistent with the accessibility of the substrate-binding site of $P450_{C\text{-}21}$ in microsomes to the polar environment. Thus, the kinetics of substrate–$P450_{C\text{-}21}$ binding in microsomes is consistent with the conclusion drawn from equilibrium binding and fluorescence-quenching data. But Kominami et al. (1986) favor the L model because, in the PC-reconstituted $P450_{C\text{-}21}$, the substrate partitioning into the lipid phase was more rapid than binding to the enzyme. In this model, substrate must gain access to the binding site on the enzyme only from the lipid phase. Their binding rate constant in the L model is two orders of magnitude lower than that reported (Griffin & Peterson, 1972) for the soluble enzyme $P450_{cam}$, which must interact with the substrate in the aqueous phase. The lower value has been attributed (Kominami et al., 1986) to higher viscosity of the lipids. The results of the present study, indicating that the polar acrylamide can compete with substrate in the microsomal membranes similar to that in the lipid-free enzyme, is inconsistent with the L model.

The most salient outcome of this study is that the substrate-binding site of $P450_{C\text{-}21}$ in the microsomes is accessible to the substrate in aqueous medium similar to that in the purified enzyme. This is consistent with the current model for the membrane topology of microsomal P450 enzymes (Sagakuchi et al., 1987; Chiarandini, et al., 1987; Nelson & Strobel, 1988; Brown & Black, 1989). In this model, a major portion of P450 molecule is exposed to the aqueous environment, suggesting that the environmental restrictions on the functionally related structural changes in microsomal P450's may be similar to those in the water-soluble enzyme. Therefore, the information on $P450_{cam}$ crystal structure (Poulos, 1986; Poulos, et al., 1987) is considered relevant to present discussion. This is because the substrate-binding site of $P450_{C\text{-}21}$ in the microsomes appears to be accessible to the highly polar molecule acrylamide and therefore to an aqueous environment. By comparing crystal structures of substrate-bound and substrate-free $P450_{cam}$, Poulos et al. (1987) showed that a camphor molecule is buried in the hydrophobic pocket completely sealed off from the external milieu. They propose that an opening which, in the crystal structure, appears to connect the molecular surface to the active site may serve as substrate access channel. In the absence of the substrate, the substrate pocket is most likely occupied by solvent molecules and the channel remains open, maintaining a solvent continuum (Poulos et al., 1987; Poulos, 1986). It is possible that a similar access channel may contribute to substrate-binding dynamics in other P450's also (Poulos et al., 1987). Perhaps the polar acrylamide binding site (tryptophan in a relatively polar environment) is in the access channel exposed to the solvent.

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Registry No. P450, 9035-51-2; acrylamide, 79-06-1; 17α-hydroxyprogesterone, 68-96-2.

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Role of the N- and C-Terminal Actin-Binding Domains of Gelsolin in Barbed Filament End Capping†

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ABSTRACT: Gelsolin is a bivalent Ca^{2+}-modulated actin-binding protein that severs, nucleates, and caps filaments. In order to gain a better understanding of the capping mechanism we have studied N- and C-terminal gelsolin fragments, 14NT and 41CT, each of which contains a single functional actin-binding site. The very tight binding measured between gelsolin and the barbed filament end requires gelsolin to greatly decrease the dissociation rate constant of the terminal actin from this end. A mechanism that could account for the observed decrease in dissociation is one in which gelsolin links two actin monomers so that they dissociate more slowly as a dimer. This cannot be the only mechanism, however, since, as shown here, 14NT and 41CT, fragments with single actin-binding sites, decrease the dissociation rate of the capped terminal actin molecule. The observations suggest that these fragments induce a conformational change in the actin monomer that either increases the affinity or alters the kinetics of the terminal actin–actin bond. The available data argue for strengthening of the terminal actin–actin bond.

Gelsolin is a Ca^{2+}-dependent actin-binding protein found in vertebrate cytoplasm (Yin & Stossel, 1979; Wang & Bryan, 1981) and blood plasma (Harris & Gooch, 1981; Nodes et al., 1987; Kwiatkowski et al., 1988). Three functional activities have been defined for this molecule. First, gelsolin forms a very tight, cooperative complex with two actin monomers in the absence of salt under conditions that will depolymerize actin filaments (Bryan & Kurth, 1984; Doi & Frieden, 1984; Coue & Korn, 1985). Second, in the presence of salt, gelsolin caps the barbed ends of actin filaments very tightly, inhibiting the addition and dissociation of monomers (Yin et al., 1981; Bryan & Coluccio, 1985). Third, free gelsolin binds laterally to filaments, weakening actin–actin interactions so that filaments break, producing capped polymers with a shorter average length (Yin et al., 1980; Wang & Bryan, 1981; Bryan & Coluccio, 1985). All of these activities require calcium binding at a regulatory site on the extreme C-terminal end of gelsolin (Yin & Stossel, 1980; Bryan & Kurth, 1984; Kwiatkowski et al., 1989).

There are three distinct actin-binding sites to carry out these activities (Bryan, 1988; Yin et al., 1988; Kwiatkowski et al., 1989), one on the C-terminal half, domains 4–6, according to the terminology of Way et al. (1989), and two on the N-terminal half of the molecule, domains 1–3. Proteolytic cleavage of the amino-terminal half separates a high-affinity G-actin-binding site, 14NT, domain 1, from an F-actin-binding site, situated on domains 2–3, whose activity is modulated by phosphoinositides (Yin et al., 1988).

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