# Quenching of Tryptophanyl Fluorescence of Bovine Adrenal P-450$_{C-21}$ and Inhibition of Substrate Binding by Acrylamide†

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ABSTRACT: Quenching of the tryptophanyl fluorescence of cytochrome P-450$_{C-21}$ by acrylamide and its relationship to substrate binding are investigated by using steady-state and time-resolved data. The average collisional quenching constant was 0.4 M whereas the quenching constant for the total fluorescence was 10.8 ± 0.9 M. This indicates that the quenching is essentially static. The quencher inhibited the binding of the substrate apparently competitively. The inhibition constant was 0.092 M, giving rise to an association constant of 10.9 M which is remarkably similar to the static quenching constant. It is suggested that tryptophan(s) may represent a key to the substrate-binding site in P-450$_{C-21}$.

Quenching of fluorescence by externally added low molecular weight compounds has been successfully used to study accessibility of fluorescent groups in proteins (Bernstein et al., 1973; Lehrer, 1975; Eftink & Ghiron, 1976; Calhoun et al., 1983). In such studies, the hydrophobic amino acid tryptophan has received considerable attention. When a fluorescent site in a protein is proximate to or in the substrate-binding site, fluorescence quenching offers an excellent spectroscopic technique to study the substrate-binding site accessibility and location within the membrane. Since tryptophanyl luminescence is environmentally sensitive, the quenching approach can also provide insight into the local environment of that region. Combined steady-state methods and time-resolved measurements of protein fluorescence [reviewed in Beecham and Brand (1985)] and separation of the fluorescence into component spectra according to quencher accessibility (Lehrer et al., 1977; Abdallah et al., 1978; Krutson et al., 1986) may provide further details about specific sites. Since the sensitivity of the fluorescence technique is high, the physical parameters and their functional correlates can be studied at equally low enzyme concentrations. This is important especially for purified membrane enzymes such as cytochrome P-450$_{C-21}$ whose tendency to aggregate at high enzyme concentrations results in altered properties (Narasimhulu et al., 1985).

The sequence of hydroxylations involved in the conversion of cholesterol to steroids with hormonal activity is well established (Eichorn & Hechter, 1958). This sequence requires interaction of intermediate steroidal substrates with specific P-450 enzymes localized in the same as well as different organelles. The manner in which the steroids are transferred to specific P-450's is not known. In addition to very high affinities of the steroids for the specific enzymes (Wilson et al., 1969; Narasimhulu, 1979; Lambeth et al., 1980; Light et al., 1981), substrate-binding site accessibility and orientation (lipid-faced or aqueous-faced?) within the membrane may be expected to play important roles. The successive hydroxylation of steroidal substrates renders the steroids progressively more polar. In studies using purified P-450$_{scc}$, cholesterol, which is highly insoluble in aqueous medium, must be presented to the enzyme in liposomes, suggesting that this substrate interacts with the binding site on the enzyme via the lipid phase (Seybert et al., 1979). Substrates of other P-450 such as P-450$_{11B}$ P-450$_{17\text{-lyase}}$, and P-450$_{C-21}$, although mainly lipid soluble, have sufficient solubility in aqueous media (Heap et al., 1970) when compared to their high affinities for the specific enzymes (Wilson et al., 1969; Narasimhulu, 1979; Nakajin et al., 1981). Therefore, depending upon the binding site accessibility, these steroids may be expected to be capable of binding to the P-450 enzyme via the aqueous or the lipid phase.

In most instances, binding of substrates to cytochrome P-450 enzymes results in a blue shift of the Soret absorption band (Narasimhulu et al., 1965; Remmer et al., 1966; Schenkman et al., 1967; Peterson et al., 1971). This shift, designated as type I spectral change (Remmer et al., 1966), is correlated with transformation of the heme from low-spin to a high-spin state (Whysner et al., 1970; Tsai et al., 1970; Stern et al., 1973; Grasdalen et al., 1975). With the use of this spectral change as the criterion for substrate binding, various aspects of binding of substrates to cytochrome P-450 enzymes have been studied (Whysner et al., 1970; Brownie & Paul, 1974; Jefcoate, 1977; Narasimhulu, 1977; Light & Orme Johnson, 1981; Narasimhulu et al., 1985). The most extensively studied is the bacterial enzyme P-450$_{cam}$ (Gunsalus & Sligar, 1978; Poulos et al., 1985). Although this is a soluble enzyme, it resembles mammalian enzymes which are membrane bound in many respects, including the substrate-binding reaction (Gotoh et al., 1983; Kominami et al., 1980). On the basis of an X-ray crystallographic study (Poulos et al., 1985), it is suggested that the substrate camphor is held in place by hydrophobic contacts with the neighboring aliphatic and aromatic residues. This implies that there are several contact points between the substrate and the hydrophobic region. Previous studies with hepatic P-450 enzymes (Al-Geilany et al., 1978; Canady et al., 1974; Marcus et al., 1985) and adrenal P-450$_{C-21}$ (Narasimhulu, 1979; Narasimhulu et al., 1985) also have shown that the binding is dominated by hydrophobic interactions. In general, the P-450 enzymes, including P-450$_{C-21}$, contain several hydrophobic residues (Gotoh et al., 1983). Although the number is not certain, the bovine adrenal P-450$_{C-21}$ contains two or more tryptophans (Hiwatashi et al., 1981; Ogishima et al., 1983).

In the present study, we have attempted to discover whether these tryptophans contribute to the substrate-binding site in the adrenal P-450$_{C-21}$. We studied the tryptophanyl fluorescence quenching by acrylamide and its relationship to substrate

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binding using both steady-state and time-resolved data.

## Materials and Methods

*Materials.* Acrylamide (4× crystallized) was a product of Serva Co., Heidelburg. Emulgen 913 was purchased from Kao Atlas Co., Tokyo. Sepharose 4B was from Pharmacia. Cholate (recrystallized), CM-Sephadex G-50, dithiothreitol, 17-hydroxyprogesterone, and *N*-acetyltryptophanamide were from Sigma.

*Purification of Cytochrome P-450$_{C\text{-}21}$.* Bovine adrenocortical microsomes were prepared as previously described (Narasimhulu et al., 1985). The microsomes were freed of sucrose and suspended in 100 mM potassium phosphate buffer containing 20% glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol (basic buffer mixture). The microsomes were then solubilized with cholate, and the P-450$_{C\text{-}21}$ was purified from the solubilized material by the procedure of Kominami et al. (1980) modified as described (Narasimhulu et al., 1985). The procedure involves hydrophobic column chromatography on aminooctylamine–Sepharose and elution with 0.2% Emulgen-containing buffer mixture.

*Detergent Removal.* The procedure has been previously described (Narasimhulu et al., 1985). It involves dialysis of the purified P-450 against 10 mM potassium phosphate buffer mixture, pH 7.25, and CM-Sephadex column chromatography. The P-450 eluted in the later more dilute fractions which were free from detectable detergent was used in the present experiments. This P-450 was stable for storage at −70 °C as well as for experiments in the absence of added detergent.

*Sodium Dodecyl Sulfate (SDS) Gel Electrophoresis.* A 5–20% gradient polyacrylamide gel electrophoresis was performed in the presence of 0.1% sodium dodecyl sulfate. The samples were boiled prior to electrophoresis.

*Spectrophotometry.* Absorption spectra of the purified P-450$_{C\text{-}21}$ were obtained with a Hitachi Model 577 split-beam spectrophotometer. Steady-state excitation and emission spectra were obtained with a Perkin-Elmer 650-10S fluorescence spectrometer in the ratio mode.

*Determination of the Apparent Substrate Dissociation Constant ($K_D^{app}$).* The substrate-produced type I difference spectrum of cytochrome P-450, characterized by a minimum at 421 nm, a 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 cytochrome. The procedure for titration with the substrate by a semi-microtitration technique has been previously described (Narasimhulu, 1979). A cuvette of 12-nm light path containing 3.0 mL of the assay system was placed in a dual-wavelength filter photometer fitted with interference filters of 421 nm ($\lambda_1$) and 407 nm ($\lambda_2$) 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-$\mu$L aliquots using a 10-$\mu$L gas-tight syringe attached to a programmable Hamilton precision dispenser. After each addition, the absorbance difference, $\Delta A$(407–421 nm), was recorded with a strip chart recorder. Addition of methanol alone was without effect. The precision of the values for the apparent $K_D$ obtained by this technique is indicated by the results of five titrations with 17$\alpha$-hydroxy progesterone, performed under identical conditions using microsomal suspensions. The 95% confidence interval obtained for $K_D$ was 6.7% of its value.

*Determination of Fluorescence Quenching Constant for Acrylamide.* Steady-state fluorescence measurements were made with a Perkin-Elmer fluorescence Model 650-10S spectrometer in the ratio mode. Unless otherwise stated, an excitation wavelength of 295 nm was used to avoid excitation of tyrosine groups. One milliliter of protein solutions in the basic buffer mixture, pH 7.25, having an optical density less than 0.05 at 295 nm was used. The temperature of the assay system was maintained at 26 °C with a circulating water bath and measured with a thermocouple. The tryptophanyl fluorescence was monitored at the emission maximum (340–342 nm) unless otherwise stated. After the initial base values of fluorescence were obtained, microliter volumes of 7 M acrylamide were added and stirred with a plastic rod. The decrease in fluorescence was recorded with a strip chart recorder. Small corrections were applied for sample dilutions caused by adding sequential aliquots of the quencher. A correction factor was also applied for the attenuation of the excitation light intensity by the added acrylamide as described by Parker (1968). This correction was made by multiplying the measured fluorescence intensity by the factor given by the equation:

$$\text{factor} = 2.3A/(1 - 10^{-A})$$

where $A$ is the measured absorbance of a given concentration of acrylamide at the exciting wavelength and 5-mm light path.

*Data Analysis.* Fluorescence quenching data were analyzed according to the (1) Stern–Volmer equation ($F_0/F = 1 + K_s[Q]$), (2) the equation $F_0/F = (1\ K_q[Q])e^{V[Q]}$ modified by Eftink and Ghiron (1976) to include the static quenching term, and (3) Lehrer's (1971) reciprocal equation ($F_0/F = 1/[Q]f_aK_q + 1/f_a$) where $K_q$ is the quenching constant and $f_a$ is the fractional maximum accessible protein fluorescence. The quenching data were fit to these equations by a search procedure developed by Dr. C. R. Eddy. The parameters were determined from the best fit to a least-squares analysis.

The substrate-binding data were analyzed as previously described (Narasimhulu et al., 1985). The substrate concentration range studied was 0.03–1 $\mu$M. On the basis of one binding site on the P-450, the bound substrate concentration was calculated by using 64 mM$^{-1}$ cm$^{-1}$ for $\Delta A$(407–421 nm). By subtracting the bound from the total substrate concentration, the free substrate concentration was determined. The $K_D^{app}$ and the maximum substrate–P-450 complex formed were calculated by using the equation:

$$[S]/[ES] = K_D^{app}/[E_t] + [S]/[E_t]$$

with a computer-assisted weighted linear regression analysis unless otherwise indicated. In this equation, S is the free substrate, ES is the complex, and $E_t$ is the total enzyme.

*Fluorescence Lifetime Measurements.* Lifetime measurements were made by using a time-correlated single photon counting instrument, exciting with the frequency-doubled pulses from a cavity-dumped dye laser pumped by a mode-locked argon ion laser. A microchannel plate photomultiplier tube and a monochromator were used for fluorescence detection. The electronics were standard nuclear instrumentation modules. The samples consisted of 0.2 $\mu$L of the protein solution in the basic buffer mixture, pH 7.25, equilibrated at 26 °C. Excitation was at 295 nm, and the fluorescence was detected at 342 nm through a polarizer set at the magic angle (54.70° from vertical). A 305-nm cutoff filter was added on the emission side to block more completely scattered light at the excitation wavelength. The instrument function was obtained with the emission monochromator set at 295 nm. Data collection, analysis, and plotting were performed by using customized routines written for the IBM-PC computer.

## Results

*Fluorescence Properties of Purified P-450$_{C\text{-}21}$.* Excitation

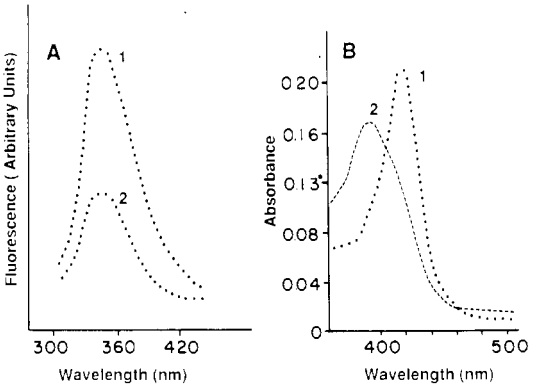


FIGURE 1: (A) Uncorrected fluorescence spectra of cytochrome P-450$_{C-21}$ in the absence (curve 1) and in the presence (curve 2) of acrylamide (0.23 M). P-450 concentration was 0.093 $\mu$M in 100 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol and 0.1 mM dithiothreitol. The temperature was 26 °C. Excitation wavelength, 295 nm. Base line obtained with the buffer alone has been subtracted. (B) Absorption spectra of cytochrome P-450$_{C-21}$ in the absence (curve 1) and in the presence (curve 2) of 17$\alpha$-hydroxyprogesterone.

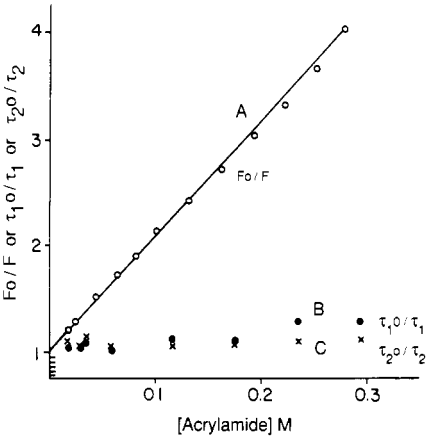


FIGURE 2: Stern–Volmer plots of acrylamide quenching of tryptophanyl fluorescence of cytochrome P-450$_{C-21}$. The assay system was 0.1 $\mu$M P-450$_{C-21}$ in 100 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol and 0.1 mM dithiothreitol. The temperature was 26 °C. The procedure was described under Materials and Methods. (○) Steady-state fluorescence quenching; (●) $\tau_1$; (×) $\tau_2$.

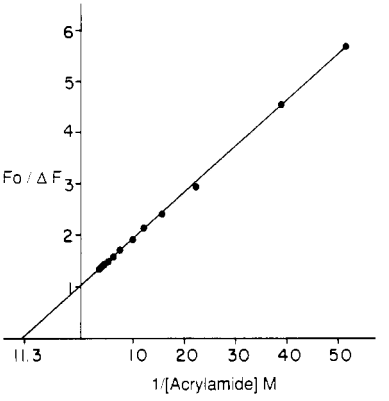


FIGURE 3: Lehrer plot ($F_0/\Delta F = 1/[Q]f_aK_a + 1/f_a$) of the steady-state fluorescence quenching data shown in Figure 2.

of purified P-450$_{C-21}$ at 295 nm resulted in an emission with a fairly broad peak at 342 nm (Figure 1). The peak position of tryptophanyl emission is generally dependent on the dielectric constant of its environment (Cowgill, 1967). From the observed peak position, it appears that this tryptophanyl environment is relatively polar with an apparent dielectric constant similar to that of methanol or propanol (Cowgill, 1967). The position of the emission peak remained constant at 342 nm when excited at different wavelengths (260–305 nm). In addition, the position of the emission peak was the same at different levels of quenching by acrylamide (Figure 1). Although this constancy of the peak position does not necessarily indicate the absence of more than one class of emitting molecules, it indicates the absence of independently emitting molecules with very different spectral properties (Galley, 1976). The constancy of the peak position when excited at different wavelengths as well as the long-wavelength broad emission would be consistent with tryptophanyl residues being exposed to a polar mobile environment (Galley, 1976).

Neither the peak position nor the spectral shape and intensity were altered by the substrate (17$\alpha$-hydroxyprogesterone). This substrate shifts the heme absorption maximum from 417 to 390 nm (Figure 1B), increasing the expected overlap with the tryptophanyl emission band. For instance, at 360 nm, a wavelength at which the tryptophanyl emission is still about 85% of the peak emission, the heme absorption increases by about 60% due to the presence of the substrate. Therefore, addition of substrate would be expected to increase any energy transfer. Since the substrate was found to have no effect on either the magnitude or the shape of the emission spectrum, it is possible that the observed fluorescence is not quenchable by energy transfer to the heme. This is surprising because in heme proteins including P-450$_{LM_2}$ (Inouye & Coon, 1985) tryptophanyl fluorescence is largely quenched by the presence of heme. Since the P-450$_{C-21}$ contains more than one tryptophan (Hiwatashi et al., 1981; Ogishima et al., 1983), it is possible that the fluorescence of only certain tryptophan(s) is quenched by the heme.

While the substrate caused a blue shift of the Soret absorption peak of the P-450, acrylamide had no effect on the absorption spectrum in the visible region.

*Quenching of Tryptophanyl Fluorescence of P-450$_{C-21}$ by Acrylamide.* Acrylamide is an efficient quencher of tryptophanyl fluorescence (Eftink & Ghiron, 1976, 1977). In general, complex formation between a fluorophore and a quencher can occur either before or after excitation. When both occur, the quenching is described by the equation:

$$F_0/F = (1 + K_{sv}[Q])(1 + K_a[Q])$$

where $K_{sv}$ and $K_a$ are the collisional and static quenching constants, respectively. The static constant is also the association constant for ground-state complex formation.

Expansion of the above equation gives

$$F_0/F = 1 + (K_{sv} + K_a)[Q] + K_{sv}K_a[Q]^2$$

When either $K_{sv}$ or $K_a$ equals zero[1]

$$F_0/F = 1 + K_a[Q] \quad \text{or} \quad 1 + K_{sv}[Q]$$

Therefore, a plot of $F_0/F$ versus [Q] will be linear when a single process is operating or dominating regardless of whether it is collisional or static quenching.

In the present experiments, the Stern–Volmer plot of the fluorescence quenching data was linear within the range of quencher concentrations tested (Figure 2, curve A). The value for the quenching constant determined from the Stern–Volmer equation using a search program was 10.8 ± 0.9 M. The Lehrer plot was also linear with a "$y$" intercept equal to 1 (Figure 3). The value (11.3 M) obtained from the "$x$" intercept for the quenching constant was within the confidence interval of the constant obtained by using the Stern–Volmer

[1] This derivation and explanation are included because in some instances, linear Stern–Volmer plots have been used as diagnostic for collisional quenching and upward curvature for static quenching.

Table I: Reversible Inhibition of 17α-Hydroxyprogesterone–P-450 Binding by Acrylamide[a]

| [steroid] (μM) | [acrylamide] (M) | [substrate–P-450 complex] (μM) obsd | calcd |
|---|---|---|---|
| 1.089 | 0 | 0.038 | 0.0365 |
| | 0.024 | 0.035 | 0.0347 |
| | 0.07 | 0.033 | 0.0326 |
| | 0.184 | 0.028 | 0.0285 |
| | 0.294 | 0.022 | 0.0253 |
| | 0.401 | 0.017 | 0.023 |
| | 0.504 | 0.012 | 0.021 |
| 11.7 | 0.504 | 0.034 | 0.0379 |

[a] The assay system consisted of purified P-450 in 100 mM potassium phosphate buffer, pH 7.25, 20% glycerol, and 0.1 mM dithiothreitol. The temperature was 26 °C. The procedure for titration and determination of the dissociation constants is described under Materials and Methods. The concentration of the substrate–P-450 complex ([ES]) was calculated from the following equation for competitive inhibition: $[ES] = [ES]_{max}/[(1 + K_d/[S])(1 + [i]/K_i)]$.

equation. Analysis of the data by weighted least-squares fit to the Scatchard equation ($r/[Q]_f = K_aN - K_ar$; $r = F/F_0$) also gave a similar value (11.6 ± 0.7 M) for the $K_a$ and indicated one binding site for acrylamide on the P-450.

Since acrylamide inhibited the binding of the substrate 17α-hydroxyprogesterone to the P-450 apparently competitively (see Inhibition of Substrate–P-450 Binding by Acrylamide), it was of interest to find out if the substrate competes with acrylamide in the fluorescence quenching reaction. The competition could be observed when the effects of the solvent (methanol) used for the steroid were taken into account. The Lineweaver–Burk plots as well as Scatchard plots were linear within the range (0.04–0.3 M) of quencher concentration tested. The substrate significantly decreased the acrylamide quenching constant while the maximum fluorescence quenched remained unaltered. However, the inhibition constant [$K_i$ = (0.31–0.67) × $10^{-6}$ M] was higher than expected [$K_i = K_d$ = (0.154–0.175) × $10^{-6}$ M] based on the acrylamide and substrate association constants. The reason for this is not clear. However, considering that acrylamide can quench the fluorescence by more than one mechanism, it is possible that there is some quenching of the tryptophanyl fluorescence even when the substrate is bound. In these studies, $[Q]_{free}$ has been assumed to be equal to $[Q]_{total}$ because $[Q]_{free} >>> [enzyme]_T$. In addition, 0.29 M was the upper limit of the quencher concentration used for the Stern–Volmer analysis of the quenching data, because at higher concentrations the reversibility of the substrate binding was decreased (Table I).

*Fluorescence Lifetime Measurements.* A collisional interaction between the fluorophore and a quencher is expected to cause reduction in the lifetime proportional to the reduction in fluorescence quantum yield. However, no reduction in lifetime is expected for static quenching (Weber, 1948). Therefore, fluorescence lifetime measurements were made in order to distinguish between dynamic and static quenching. A representative example of fluorescence decay is shown in Figure 4. The decay curve could be best described by a sum of three exponential functions (with $\chi^2$ values usually ranging from 0.8 to 1.2). In the absence of the quencher, the three lifetimes obtained by averaging values from three experiments were 1.6, 6.9, and 0.32 ns. Their fractional contributions to the total fluorescence were 45%, 34%, and 20%, respectively. Acrylamide caused little or no decrease in the lifetimes. The $\tau_1$ value (1.6 ns) decreased the most, and a value of 0.9 M for the collisional quenching constant was estimated from the data shown in Figure 2 (curve B) by a linear least-squares calculation. The $K_{sv}$ for $\tau_2$ (6.9 ns) was similarly estimated to be

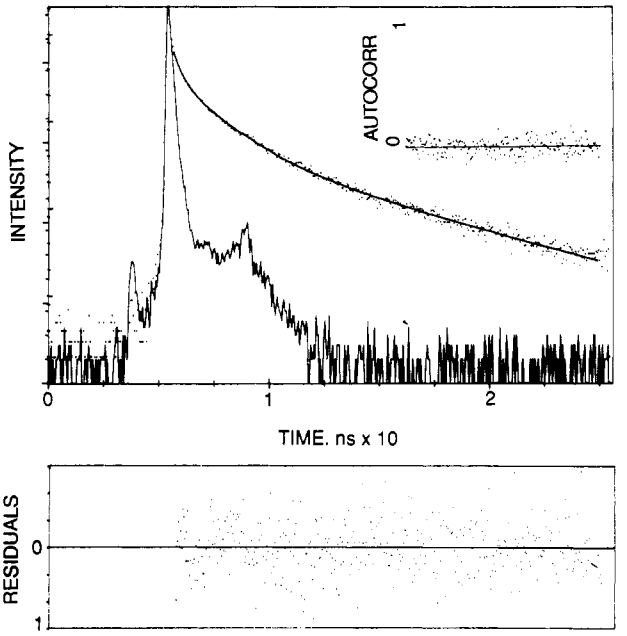


FIGURE 4: Representative fluorescence decay data. The procedure was described under Materials and Methods. The P-450–buffer mixture described under Figure 1 contained 0.28 M acrylamide. Excitation at 295 nm; emission at 340 nm. The solid line is the computer best fit of the data. The lifetimes were $\tau_1$ = 1.525 ns, $\tau_2$ = 5.858 ns, and $\tau_3$ = 0.282 ns. $\chi^2$ = 0.9824, and standard deviation = 1.0355 ± 0.9631.

0.01 M. Since the $P\text{-}450_{C\text{-}21}$ contains more than one tryptophan, the Stern–Volmer plots (Figure 2, curves B and C) were constructed assuming that the different lifetimes represent different chromophores. A composite $K_{sv}$ of 0.4 M was obtained by taking the fractional contributions into account. Further experiments are needed to determine whether the very short lifetime component originates from residual scattered light, a third heme-quenched component, or both. However, for the present purpose of determining the contribution by dynamic quenching, Stern–Volmer analysis of the decreases in $\tau_1$ and $\tau_2$ is likely sufficient since $\tau_3$ is a minor component that does not contribute significantly to the average lifetime (3.13 ns) calculated via fractional intensity contributions.

*Inhibition of Substrate–P-450 Binding by Acrylamide.* The Lineweaver–Burk plots (1/[ES] vs 1/[S]) of the substrate (17α-hydroxyprogesterone)-binding data obtained in the absence and in the presence of acrylamide were linear. Acrylamide inhibited the substrate binding as indicated by the increase in the apparent substrate dissociation constant (from 0.164 to 0.584 μM) obtained by least-squares calculations. The inhibitor was without significant effect on the maximum level of substrate–P-450 complex formed. These results would be consistent if acrylamide competes with the substrate for the same binding site on the enzyme. Therefore, the binding data were analyzed by the equation for competitive inhibition:

$$\frac{1}{[ES]} = \frac{1}{[ES]_{max}} + \frac{1}{[ES]_{max}}\left[K_d + \frac{K_d[i]}{K_i}\right]\frac{1}{[S]}$$

where ES is the substrate–P-450 complex and [i] is the inhibitor concentration. $K_i$ and $K_d$ are the dissociation constants for the inhibitor and the substrate, respectively. The inhibition constant ($K_i$) was calculated from the following equation for the $x$ intercept; that is, when 1/[ES] = 0.

$$K_i/(K_dK_i + K_d[i]) = 1/[S]$$

The value obtained for $K_i$ was 0.092 M. This gives a weak equilibrium association constant ($1/K_i$) for the inhibitor of 10.9

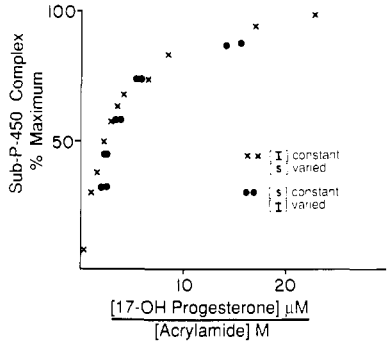


FIGURE 5: Competition between 17$\alpha$-hydroxyprogesterone and acrylamide in the substrate–P-450 binding reaction. Experimental conditions were as described in Table I. (×) [Acrylamide], [I] constant, [17$\alpha$-hydroxyprogesterone], [S], varied; (●) [S] constant, [I] varied.

M. The inhibition could, of course, be reversed by adding more substrate (Table I). The magnitudes of the observed inhibition by acrylamide and reversal by adding more substrate were very similar to the values calculated based on the equation for competitive inhibition. The inhibition depended on the substrate:inhibitor ratio (Figure 5) rather than the inhibitor concentration. Since the equation for inhibition of an enzyme–substrate reaction by binding of an inhibitor to the substrate is identicial, in form, with the normal equation for competitive inhibition (Dixon & Webb, 1979), it was important to find out if acrylamide binds to substrate, 17$\alpha$-hydroxyprogesterone. Therefore, using the tritiated steroid, we determined the effect of acrylamide on the partitioning of the steroid between dichloromethane and water at the inhibitor:substrate concentration ratio needed for inhibiting the steroid–P-450 binding reaction as well as at higher ratios (2×). It was found to be without effect as indicated by the lack of increase in the radioactivity recovered in the aqueous phase, by the presence of acrylamide. Since its solubility in water is about 100 times that in dichloromethane, the aqueous phase would be expected to contain nearly all of the added acrylamide, under the present experimental conditions. Since the solubility of the steroid in water is negligible relative to that in the organic solvent, most of the steroid would be in the organic phase unless it binds to acrylamide. The radioactivity of the aqueous phase was unaltered by the presence of acrylamide. In addition, a mathematical model (Dixon & Webb, 1979) in which the inhibitor forms a complex with the substrate and the observed $K_I$ assumed to be its dissociation constant did not fit the data. This is also consistent with the affinity of the steroid for the P-450 being 5 orders of magnitude higher than for the inhibitor. Therefore, it is concluded that acrylamide does not bind to the substrate and that it inhibits the substrate–P-450 binding by competing with the substrate for the same site on the enzyme. Scatchard analysis of the substrate-binding data indicated one binding site.

## Discussion

The results of fluorescence experiments indicate that the observed tryptophanyl fluorescence of cytochrome P-450$_{C\text{-}21}$ is completely accessible to quenching by acrylamide. This is evidenced by the $y$ intercept of the Lehrer plot being equal to 1. The quenching is mostly static as evidenced by the dynamic component being negligibly small. The average collisional quenching constant estimated from lifetime data was 0.4 M. This value is within the error range of determining the overall quenching constant (10.8 ± 0.9 M) by steady-state fluorescence measurements. This result indicates that essentially one type of quenching, static quenching, is involved, which explains the linearity observed for the Stern–Volmer plot. It appears that linear Stern–Volmer plots observed for several other proteins have been interpreted in terms of purely collisional quenching and static quenching is considered only when the plot curves upward (Eftink & Ghiron, 1981). In addition, on the basis of acrylamide quenching studies using several proteins, it has been concluded that acrylamide does not interact significantly with proteins and that the quenching is mostly dynamic (Eftink <<ammp Ghiron, 1981). The fact that this generalization does not hold for the P-450$_{C\text{-}21}$ makes this enzyme system a rare example of a linear Stern–Volmer plot although the quenching is essentially static. That the linear Stern–Volmer plot can be either purely collisional or static is indicated by the equations derived under Quenching of Tryptophanyl Fluorescence of P-450$_{C\text{-}21}$ by Acrylamide which are consistent with the well-known original theory of Stern–Volmer. Since the Stern–Volmer plot was linear even at the quencher concentrations at which the $F_0/F = e^{v[Q]}$ (Eftink & Ghiron, 1976, 1981) would be nonlinear their sphere a model is not applicable to this enzyme system. The Lehrer (1971) plot which is analogous to the usual reciprocal-binding plot was also linear. These results indicate that the reaction of acrylamide with the fluorophore is an equilibrium binding reaction and the quenching constant (10.8 ± 0.9 M) represents the association constants ($K_a$) for the quencher.

The results of substrate-binding studies indicated that acrylamide inhibited the binding of the substrate 17$\alpha$-hyrdroxyprogesterone to the cytochrome P-450$_{C\text{-}21}$. The inhibition data could be best described by the classical equation for competitive inhibition. Accordingly, the degree of inhibition depended upon the inhibitor:substrate ratio rather than the inhibitor concentration, and addition of sufficient substrate reversed the inhibition. These results may be interpreted as being due to (1) competition between the inhibitor and the substrate for a site on the enzyme, (2) binding of the inhibitor to the substrate, thereby decreasing the free substrate concentration, and (3) allosteric interactions. Since the steroid was not capable of binding to acrylamide, the observed inhibition is not due to binding of the inhibitor to the substrate. Since acrylamide is not a structural analogue of the substrate, allosteric interactions must be considered. If the substrate and the inhibitor bound to different sites on the enzyme in such a way that the binding of either one caused a conformational change in the enzyme which prevented the other from binding, the inhibition could be (but not necessarily) competitive (Monod et al., 1963). However, neither acrylamide nor the steroid altered the wavelength of the tryptophanyl emission peak, indicating the absence of such conformational changes which can alter the tryptophanyl environment. That tryptophan(s) may represent the binding site for the steroid as well as acrylamide is indicated by (1) the number of binding sites being 1 in both cases, (2) the recprocal of the acrylamide inhibition constant ($1/K_i$ = 10.9 M) for substrate binding being equal to its fluorescence quenching constant (10.9 ± 0.9 M), and (3) the competition between the substrate and the inhibitor in the fluorescence quenching reaction as well. These results also indicate that the observed inhibition is probably due to the steroid and acrylamide competing for the same site on the enzyme.

The emerging picture is as follows: Since the hydrophobic interactions play a major role in the binding of substrates to the P-450 enzymes (White & Coon, 1981), the P-450$_{C\text{-}21}$ has a hydrophobic pocket containing at least one of the tryptophans. This pocket is designed for the binding of the substrate with high specificity and affinity. Since substrate binding

results in a conformationally driven spin-state transition of the heme and the binding of acrylamide is without effect on the spin state, the substrate must make specific contacts with more residues than acrylamide in the pocket. Since the tryptophanyl environment may be relatively polar (as judged from the position of the emission peak) and acrylamide, which competitively inhibits substrate binding, is a highly polar molecule, the pocket may also contain polar regions crucial for substrate binding. This is consistent with the requirement for the 3- and 20-carbonyl and 17$\alpha$-hydroxy groups in the steroid molecule to display high affinity to the P-450 (Rosenthal & Narasimhulu, 1969). Since acrylamide and the substrate may compete for the same site and acrylamide has to bind to tryptophans to statically quench the fluorescence, at least one of the tryptophans in the P-450 must contribute to a crucial point of contact for the substrate in the hydrophobic pocket.

Such a picture of a hydrophobic pocket having several contact points for the substrate is consistent with those which have emerged in the case of other bacterial (Poulos et al., 1985) as well as mammalian (Kunze et al., 1983) P-450 enzymes. Most precise information available comes from crystallographic study of substrate–P-450$_{cam}$ complex (Poulos et al., 1985). As indicated earlier, in P-450$_{cam}$, the substrate, camphor, is held in place by hydrogen bonding as well as hydrophobic contacts between the substrate and neighboring aliphatic and aromatic residues. The involved residues are tyrosine-96, leucine-244, and valine-247 (Poulos et al., 1985). Sequence alignment of different P-450 enzymes (Gotoh et al., 1983) indicates that none of these regions is conserved. Therefore, it is suggested (Poulos et al., 1985) that these very regions may be the primary variable sites in different P-450 enzymes with differing substrate specificities. Therefore, tryptophan(s) may well represent a key to the substrate-binding site in cytochrome P-450$_{C\text{-}21}$.

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**Registry No.** $H_2C{=}CHCONH_2$, 79-06-1; cytochrome P-450$_{C\text{-}21}$, 9035-51-2; L-tryptophan, 73-22-3; 17$\alpha$-hydroxyprogesterone, 68-96-2.

## REFERENCES

Abdallah, M. A., Biellmann, J. F., Wiget, P., Joppich-Kuhn, R., & Luisi, P. L. (1978) *Eur. J. Biochem. 89*, 397–405.

Al-Gailany, K. A. S., Houston, J. B., & Bridge, J. W. (1978) *Biochem. Pharmacol. 27*, 783–788.

Beechem, J. M., & Brand, L. (1985) *Annu. Rev. Biochem. 54*, 43–71.

Brownie, A. C., & Paul, D. P. (1974) *Endrocr. Res. Commun. 1*, 321–330.

Burnstein, E. A., Vedenkina, N. S., & Ivkova, M. N. (1973) *Photochem. Photobiol. 18*, 63–279.

Calhoun, D. B., *Vanderkooi*, J. M., & Englander, S. W. (1983) *Biochemistry 22*, 33–1539.

Canady, W. J., Robinson, D. A., & Colby, H. D. (1974) *Biochem. Pharmacol. 23*, 3075.

Cinti, D. L., Sligar, S. G., Gibson, G. G., & Schenkman, J. B. (1979) *Biochemistry 18*, 37–42.

Cowgill, R. W. (1967) *Biochim. Biophys. Acta 133*, 6–18.

Dixon, M., & Webb, E. C. (1979) *The Enzymes*, pp 334–359, Academic Press, New York.

Ebel, R. E., O'Keefe, D. H., & Peterson, J. (1978) *J. Biol. Chem. 255*, 3888–3897.

Eftink, M. R., & Ghiron, C. A. (1976a) *Biochemistry 15*, 672–680.

Eftink, M. R., & Ghiron, C. A. (1976b) *J. Phys. Chem. 80*, 486–493.

Eftink, M. R., & Ghiron, C. A. (1977) *Biochemistry 16*, 5546–5551.

Eftink, M. R., & Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199–217.

Eichhorn, J., & Hechter, O. (1958) *Proc. Soc. Exp. Biol. Med. 97*, 614.

Galley, W. C. (1976) *Biochem. Fluoresc.: Concepts 2*, 409–439.

Gotoh, O., Tagashira, Y., Suzuki, T., & Fujii-Kuriyama, Y. (1983) *J. Biochem. (Tokyo) 93*, 807–817.

Grasdalen, H., Blackstrom, D., Ericksson, L. E. G., Ehrenberg, A., Moldeus, P., Von Bahr, C. V., & Orrenuis, S. (1975) *FEBS Lett. 60*, 274–279.

Gunsalus, I. C., & Sligar, S. G. (1978) *Adv. Enzymol. Relat. Areas Mol. Biol. 47*, 1–44.

Hanukoglu, I., Privalle, C. T., & Jefcoate, C. R. (1981) *J. Biol. Chem. 256*, 4329–4335.

Heap, R. B., Symons, A. M., & Watkins, J. C. (1970) *Biochim. Biophys. Acta 218*, 482–495.

Hiwatashi, A., & Ichikawa, Y. (1981) *Biochim. Biophys. Acta 664*, 33–48.

Inouye, K., & Coon, M. J. (1985) *Biochem. Biophys. Res. Commun. 128*, 676–682.

Jefcoate, C. R. (1977) *J. Biol. Chem. 252*, 8788–8796.

Jefcoate, C. R. (1982) *J. Biol. Chem. 257*, 4731–4737.

Knutson, J. R., Davenport, L., Beechem, J. M., Walbridge, D. G., & Brand, L. (1986) in *Excited State Probes in Biochemistry and Biology* (Szabo, A. G., & Massotti, L., Eds.) Plenum, New York.

Kominami, S., Ochi, H., Kobayashi, Y., & Takemori, S. (1980) *J. Biol. Chem. 225*, 3386–3394.

Kunze, K. L., Mangold, B. L. K., Wheeler, C., Beilan, H. S., & Ortiz De Montellani, P. R. (1983) *J. Biol. Chem. 258*, 4202–4207.

Lambeth, J. D. (1981) *J. Biol. Chem. 256*, 4657–4762.

Lambeth, J. D., Kamin, H., & Seybert, D. W. (1980) *J. Biol. Chem. 255*, 8282–8288.

Lehrer, S. S. (1971) *Biochemistry 10*, 3254.

Lehrer, S. S. (1975) in *Concepts in Biochemical Fluorescence* (Chen, R., & Edelhock, H., Eds.) Marcel Dekker, New York.

Light, D. R., & Orme-Johnson, N. R. (1981) *J. Biol. Chem. 256*, 3433–3450.

Monod, J., Changeux, J. P., & Jacob, F. (1963) *J. Mol. Biol. 6*, 306.

Narasimhulu, S. (1971) *Arch. Biochem. Biophys. 147*, 391–404.

Narasimhulu, S., Cooper, D. Y., & Rosenthal, O. (1965) *Life Sci. 4*, 2101–2107.

Narasimhulu, S., Eddy, C. R., Dibartolomeis, M., Kowluru, R., & Jefcoate, C. R. (1985) *Biochemistry 24*, 4287–4294.

Ogishima, T., Okada, Y., Kominami, S., Takemori, S., & Owara, T. (1983) *J. Biochem. (Tokyo) 94*, 1711–1714.

Parker, C. A. (1968) *Photoluminescence of Solutions*, pp 220–222, Elsevier, New York.

Paul, D. P., Gallant, S., Orme-Johnson, N. R., & Browne, A. C. (1976) *J. Biol. Chem. 251*, 7120–7126.
Poulos, T. L., Finzel, B. C., Gunsalus, I. C., Wagner, G. G., & Kraut, J. (1985) *J. Biol. Chem. 260*, 16122–12130.
Peterson, J. A. (1971) *Arch. Biochem. Biophys. 144*, 678–686.
Remmer, H., Schenkman, J. B., Estabrook, R. W., Sasame, H., Gillette, J. R., Narasimhulu, S., Cooper, D. Y., & Rosenthal, O. (1966) *Mol. Pharmacol. 2*, 187–196.
Ristau, O., Rein, H., Janig, G. R., & Ruckpaul, K. (1978) *Biochim. Biophys. Acta 536*, 226–234.
Rosenthal, O., & Narasimhulu, S. (1969) *Methods Enzymol. 15*, 596–637.
Schenkman, J. B., Remmer, H., & Estabrook, R. W. (1967) *Mol. Pharmacol. 3*, 113–121.
Seybert, D. W., Lancaster, J. R., Lambeth, J. D., & Kamin, H. (1979) *J. Biol. Chem. 254*, 12088–12098.
Stern, J. O., Peisach, J.,. Blumberg, W. E., Lu, A. Y. H., & Levin, W. (1973) *Arch. Biochem. Biophys. 156*, 404–416.
Tasai, R. L. Y. C. A., Gunsalus, I. C., Peisach, J., Blumberg, W. H., & Beinert, H. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 1157–1163.
Weber, G. (1948) *Trans. Faraday Soc. 44*, 1985.
White, R. E., & Coon, M. J. (1980) *Annu. Rev. Biochem. 49*, 315–355.
Whysner, J. A., Ramsayer, J., & Harding, B. W. (1970) *J. Biol. Chem. 245*, 5441–5449.
Wilson, L. D., Oldham, S. B., & Harding, B. W. (1969) *Biochemistry 8*, 2975–2980.

# Conjugation to an Antifibrin Monoclonal Antibody Enhances the Fibrinolytic Potency of Tissue Plasminogen Activator in Vitro†

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ABSTRACT: Tissue plasminogen activator (tPA) was covalently linked by disulfide bonds to a monoclonal antibody specific for the amino terminus of the $\beta$ chain of fibrin (antibody 59D8). The activity of the tPA–59D8 conjugate was compared with that of tPA, urokinase (UK), and a UK–59D8 conjugate. For lysis of fibrin monomer, tPA was 10 times as potent as UK, whereas both UK–59D8 and tPA–59D8 conjugates were 100 times as potent as UK and 10 times as potent as tPA. Conjugation of tPA or UK to antibody 59D8 produced a 3.2–4.5-fold enhancement in clot lysis in human plasma over that of the respective unconjugated plasminogen activator. However, the UK–59D8 conjugate was only as potent as tPA alone. Antibody-conjugated tPA or UK consumed less fibrinogen, $\alpha_2$-antiplasmin, and plasminogen than did the unconjugated activators, at equipotent fibrinolytic concentrations. Antibody targeting thus appears to increase the concentration of tPA in the vicinity of a fibrin deposit, which thereby leads to enhanced fibrinolysis.

The use of thrombolytic agents in the treatment of acute myocardial infarction is receiving increasing attention because of recent reports that both mortality and morbidity can be reduced by their early administration (GISSI, 1986; TIMI, 1985). However, the frequency of hemorrhagic complications resulting from this form of therapy has prompted investigators to seek more specific agents.

The principle of antibody targeting of cytotoxic agents to cell-surface antigens has been explored extensively (Letvin et al., 1986; Marsh & Neville, 1986; Ramakrishnan & Houston, 1985; Vitetta & Uhr, 1985). The proposed advantage is the selective ablation of a subset of cells identified by the antibody. We reasoned that the same concept could be applied to the directing of lytic agents; in particular, we sought to improve the specificity of tissue plasminogen activator (tPA).[1] Our laboratory has already demonstrated that a covalent (disulfide) complex of murine fibrin-specific antibody (antibody 64C5) and urokinase (UK) is 100 times more efficient than UK alone in an in vitro fibrinolytic system (Bode et al., 1985). The chemical coupling strategy employed at that time, however, was limited by poor yields of functional, coupled urokinase. Here we used a chemical coupling strategy that entails modification of the antibody with 2-iminothiolane and formation of a disulfide bond between it and the plasminogen activator (Runge et al., 1987). This strategy produces conjugates in yields sufficient for definitive testing. The fibrinolytic potency and specificity of conjugates of antifibrin monoclonal antibody 59D8 and either tPA or UK were enhanced in comparison with those of the unconjugated plasminogen activators.

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[1] Abbreviations: 59D8, antifibrin monoclonal antibody 59D8; S-2288, chromogenic substrate H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide dihydrochloride; S-2251, H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride; PBSA, phosphate-buffered saline azide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPDP, succinimidyl 3-(2-pyridyldithio)propionate; tPA, tissue plasminogen activator; UK, urokinase; Tris, tris(hydroxymethyl)aminomethane.

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