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A FLUORESCENCE STUDY OF THE INTERACTIONS OF BENZO[*a*]PYRENE, CYTOCHROME P450c AND NADPH–CYTOCHROME P450 REDUCTASE

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Abstract—Fluorescence quenching of benzo[*a*]pyrene (BP) by cytochrome P450c was used to probe this substrate–enzyme binding interaction. Addition of NADPH–cytochrome P450 reductase, an essential electron carrier during P450 catalysis, resulted in increased quenching and thus strengthened binding of BP to P450c. This shows that the role of reductase extends beyond that of an electron transfer agent to influence substrate binding. Fluorescence titration measurements revealed that reductase and P450c formed a complex with an apparent K_D of 13.7 ± 0.9 nM. Reductase had no effect in the presence of an anti-P450c monoclonal antibody which inhibits BP hydroxylation, which suggests that this monoclonal antibody binds P450c near its reductase binding region.

The cytochromes P450 are a family of enzymes that catalyze the oxidation of a wide array of lipophilic compounds [1, 2]. These include xenobiotics such as drugs and carcinogens, as well as endogenous compounds such as prostaglandins, fatty acids and steroids. The various forms of P450† differ in their substrate and product specificities and reactivities. This variation in substrate recognition arises from amino acid sequence differences in an active site region comprised of the heme and substrate binding sites. While this region confers substrate specificity, other undefined regions on the P450 surface are also functionally active as they contain binding sites for essential catalytic cofactors such as NADPH–cytochrome P450 reductase [3].

The heme group has served as an excellent internal spectral probe in many conformational studies on hemeproteins. For the polyaromatic hydrocarbon-metabolizing rat liver microsomal P450c (corresponding to IA1 [4]), binding to the substrate benzo[*a*]pyrene (BP) results in excitation energy transfer from this fluorescent donor to the P450c heme acceptor. This property has proven useful as a probe for BP binding to P450c [5, 6]. In this report the effect of reductase and an inhibitory monoclonal antibody to P450c was thus examined in order to evaluate whether their interaction with the P450c surface is coupled with a distal interaction, that of BP with the substrate binding site of P450c.

MATERIALS AND METHODS

P450c and P450b were purified from liver microsomes of polychlorinated biphenyl-treated male rats as described [7] and stored in 50 mM potassium phosphate buffer (pH 7.25) containing 60% glycerol at −80°. The concentration of P450 was determined from the CO-difference spectrum [8], and its protein concentration was measured by the BCA assay (Pierce Chemical Co., Rockford, IL). The specific content and maximal wavelength of the CO-difference spectrum of P450c were 14.6 nmol/mg protein and 447.6 nm, respectively. The purified P450c was also homogeneous on sodium dodecyl sulfate–polyacrylamide gel electrophoresis [9]. NADPH–cytochrome P450 reductase was purified as described [10, 11], and its concentration was determined from its absorbance spectrum [12]. Monoclonal antibodies 1-7-1 to P450c [13] and 1-98-1 to P450j [14] were purified from mouse ascites fluid [15].

The interaction between BP and P450c was examined after preincubating these components at about 50-fold their final concentrations, at 25° for 30 min. The stock solution of BP (recrystallized, from the Sigma Chemical Co., St. Louis, MO) was prepared in dimethyl sulfoxide (DMSO), whose concentration in the incubation mixture was under 5%. Then dilauroyl-L-3-phosphatidylcholine (DLPC; Serdary Research Laboratories, London, Ontario, Canada) suspended in 50 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol was added in a ratio of 150 μg/nmol P450c and incubated at 25° for an additional 30 min. When present, reductase was added with DLPC. For experiments with monoclonal antibody, P450c was preincubated with antibody at 25° for 30 min before addition of BP. The mixture was then diluted to the desired concentration with 0.1 M potassium phosphate buffer (pH 7.25) containing 20% glycerol.

Fluorescence emission spectra and anisotropies were determined at 25° with an SLM 8000c photon counting spectrofluorometer. For fluorometric titrations, excitation and emission wavelengths were

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† Abbreviations: P450, cytochrome P450; reductase, NADPH–cytochrome P450 reductase; BP, benzo[*a*]pyrene; DLPC, dilauroyl-L-3-phosphatidylcholine; and MAb, monoclonal antibody.

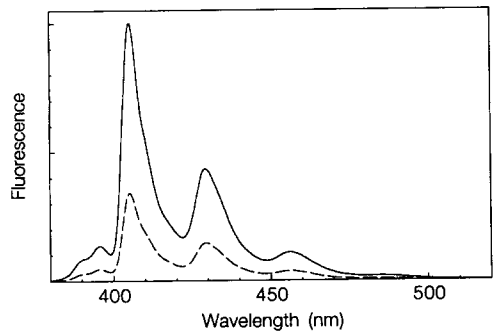

Fig. 1. Fluorescence emission spectra of benzo[*a*]pyrene in the absence (——) and presence (– –) of P450c. The solution contained BP (30 nM), P450c (100 nM), and DLPC (15 μg/mL) in 0.1 M potassium phosphate buffer (pH 7.25) containing 20% glycerol. Excitation was at 368 nm. Excitation and emission slits were 4 and 2 nm, respectively.

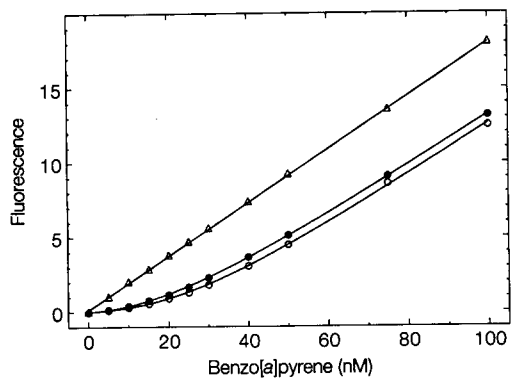

Fig. 2. Fluorescence titration of P450c with benzo[*a*]pyrene. Different amounts of BP were in buffer (△) or mixed with 50 nM P450c with (○) or without (●) 147 nM reductase. Fluorescence emission intensity at 405 nm was measured with excitation at 388 nm. Excitation and emission slits were 2 and 4 nm, respectively.

388 and 405 nm, respectively. The corresponding slit widths were 2 and 4 nm.

RESULTS

Since heme is a powerful quencher of fluorescence, this property is a useful probe for monitoring binding of fluorescent substrates or ligands to hemeproteins such as P450c. The fluorescence intensity of BP was decreased upon addition of P450c (Fig. 1). This BP-450c interaction was further examined in fluorescence titration experiments (Fig. 2). In the absence of P450c, fluorescence increased linearly with BP, whereas in its presence the slope of the titration curve was initially low and increased gradually to that found in the absence of P450c. When NADPH–cytochrome P450 reductase was also present, a similar curve was observed, although with lower fluorescence. In a control experiment using another P450 form, P450b, BP fluorescence was not quenched

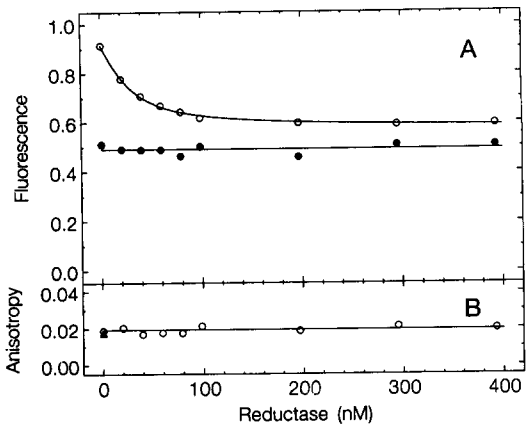

Fig. 3. Effect of NADPH–cytochrome P450 reductase on fluorescence of BP–P450c. (A) Fluorescence intensity was measured in the absence (○) and presence (●) of MAb 1-7-1. Different amounts of reductase were added to the mixture of 100 nM P450c, 20 nM BP, 15 μg/mL DLPC and 40 μg/mL MAb 1-7-1. (B) Anisotropy was measured in the presence of different amounts of reductase (○). The anisotropy for BP alone is also shown (▲).

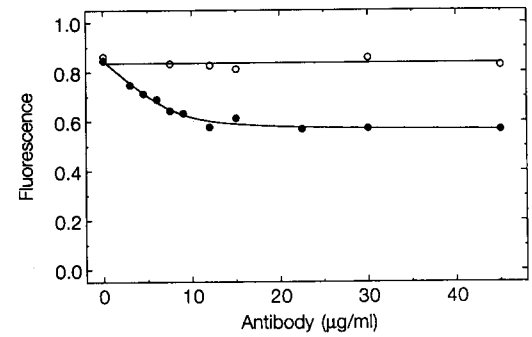

Fig. 4. Effect of anti-P450 antibody on the fluorescence of BP-P450c. Different amounts of antibody were preincubated with 100 nM P-450c, and then 20 nM BP and 15 μg/mL DLPC were added. Measurements were performed using MAb 1-7-1 to P-450c (●) and MAb 1-98-1 to P-450j (○).

(data not shown). The quenching thus is a probe for a specific interaction between BP and P450c.

We examined in more detail the effect of NADPH–cytochrome P450 reductase on the fluorescence of the BP–P450c system. Added reductase resulted in a maximal 35% decrease in fluorescence intensity (Fig. 3A), which indicates that reductase binding to P450c promotes the association of BP and P450c. In the absence of P450c, reductase had no effect on BP fluorescence. Least-squares analysis of the titration curve revealed an apparent K_D of 13.7 ± 0.9 nM for the interaction of reductase with BP–P450c.

The effect of MAb 1-7-1, which inhibits the BP hydroxylase activity of P450c [13], was also assessed. In the presence of this MAb, the fluorescence of the BP–P450c system was maximally decreased by 45% (Fig. 4). The apparent K_D of this interaction was

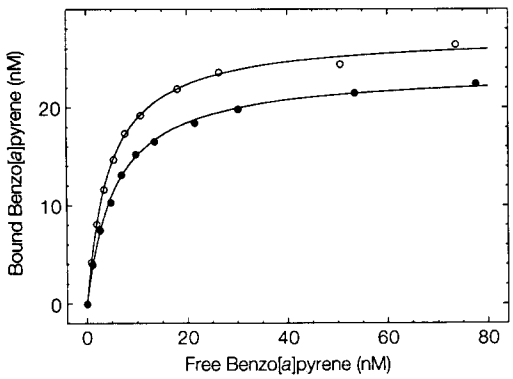

Fig. 5. Effect of NADPH–cytochrome P450 reductase on the binding of BP to P450c. The amounts of bound and free BP were calculated from the fluorescence data in Fig. 2, in the presence (○) and absence (●) of reductase.

5.3 ± 1.0 nM. The effect of MAb 1-7-1 is specific since MAb 1-98-1, which recognizes P450j [14], had no effect on fluorescence quenching of BP by P450c. The combined effect of MAb 1-7-1 and reductase was also examined: the MAb completely prevented reductase-induced fluorescence quenching (Fig. 3A).

To quantitatively evaluate the binding data of Fig. 2, the efficiency of energy transfer between BP and heme must be known. From calculations involving the large spectral overlap between BP fluorescence and heme absorbance [6], the critical distance for 50% energy transfer, R_0, is 44 Å. From the standard distance–efficiency relationship [16], one can thus calculate that transfer is essentially complete (>95%) for a BP-heme distance of less than 27 Å, which is much greater than the P450 heme–substrate distance of about 4 Å [17]. This high efficiency was confirmed experimentally by fluorescence lifetime measurements of BP in the absence and presence of P450c, which indicated an efficiency greater than 98% (unpublished data, Omata Y, Friedman FK and Knutson JR, National Institutes of Health). In addition, a mixture of BP and P450c, or BP and P450c in the presence of various amounts of reductase, had the same fluorescence anisotropy as free BP (Fig. 3B). Since P450c-bound BP is partially immobilized and would thus have a higher anisotropy than free BP, this result also indicates that the observable fluorescence derives from free BP, and that the fluorescence of bound BP is undetectably low.

Since free BP is thus proportional to fluorescence, the amounts of free and bound P450c in Fig. 2 are calculable. When the titration data were thus plotted, a greater fluorescence change in the presence of reductase was evident (Fig. 5). The apparent K_D for the BP–P450c interaction was 5.8 ± 0.2 and 4.6 ± 0.2 nM in the absence and presence of reductase, respectively.

DISCUSSION

A basic question in enzyme structure–function studies concerns the coupling between the conformation of the substrate binding site and binding of functional modulators to distal regions of the protein. In this study we examined the effect of one such modulator, NADPH–cytochrome P450 reductase, on the BP–P450c interaction. Fluorescent quenching of BP upon binding to P450c [5, 6, 18] was thus used to measure the extent of association. The data show that BP fluorescence was essentially completely quenched by P450c, thus greatly facilitating determination of the binding parameters.

Binding of substrates to P450s has often been monitored by utilizing the substrate-induced heme spectral changes [19]. These changes are not directly attributable to substrate binding but rather derive from perturbation of the iron spin-state equilibrium. Substrate binding isotherms based on absorbance changes are therefore not simply related to substrate binding, but depend on the low/high spin equilibria for both substrate-bound and substrate-free P450, as well as on the substrate equilibria with low and high spin P450 [20]. Thus, if a given substrate binds equally well to P450 in both spin states, no absorbance change will be observed, which can be misinterpreted to mean that no binding occurs. Fluorescence energy transfer, on the other hand, directly measures substrate–P450 association irrespective of spin state, thus greatly simplifying binding analysis.

NADPH–cytochrome P450 reductase is an essential component of the mixed-function oxygenase system that specifically binds to P450 and transfers an electron to the heme iron during the catalytic cycle [3, 21, 22]. Several studies have established that reductase associates with purified P450 to form a catalytically active complex [23–30]. Our data indicate that the K_D for complex formation is 13.7 nM, a value in agreement with that found by other methods for other forms of P450 [31, 32].

A protein–protein association such as that of reductase–P450 necessarily alters the energetics and dynamics of the complementary binding surfaces. It is not established whether such changes generally remain localized or whether they are transmitted to more remote regions such as an active site. Since reductase increased binding of BP to P450c, binding of reductase to its receptor region on the P450c surface indeed transmits a change to the interior of the P450c molecule. This result shows that reductase function extends beyond its conventional role as an electron carrier. It is intriguing to consider the extent of the reductase-induced changes in P450c: whether these are global, or correspond to a channel of localized changes between P450c surface and substrate binding site, similar to that of an electron transfer pathway.

Since the interactions of P450 with reductase and substrate have often been considered individually, it is unclear to what extent these two interactions are coupled. Reductase binding perturbs the P450 heme, as evidenced by changes in magnetic circular dichroism [25], and substrate binding influences the heme iron spin state [33–35]. Previous evidence for some coupling between reductase and substrate binding to P450 forms other than the P450c described in this report include substrate-induced acceleration

of reductase-mediated reduction of ferric heme [36–38], a finding attributed to substrate-induced changes in the reduction potential of the iron [34, 39].

We also examined the effect of MAb 1-7-1, which specifically inhibits BP hydroxylation by P-450c [13]. The addition of MAb increased the amount of fluorescence quenching, which implies that the MAb enhances binding of BP to P450c. This MAb thus inhibits catalytic activity by a mechanism other than inhibition of substrate binding. The MAb inhibition of reductase-induced fluorescence quenching indicates that the MAb diminishes the interaction of P450c with reductase, and thus may inhibit catalytic activity by interfering with reductase-mediated electron transfer. The simplest explanation for the effect of MAb is that it binds to the reductase receptor region on the P450c molecule and sterically prevents reductase binding. Alternatively, the MAb may bind elsewhere but induce a conformational change in the reductase binding region which weakens or abolishes its complementary interaction with reductase.

In summary, NADPH–cytochrome P450 reductase promoted binding of BP to P450c, as reflected in enhanced fluorescence quenching. The action of reductase thus extends beyond its well-known and essential role as an electron carrier, and suggests a role in substrate–P450 interaction.

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