Binding of Reactive Metabolites of Aromatic Hydrocarbons to Specific Microsomal Proteins*

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Many toxic and/or carcinogenic compounds exert their adverse effects only after metabolic activation. 1 The reactive metabolites thus formed can bind covalently to cellular macromolecules and disturb normal cellular functions, giving rise to cytotoxic effects, mutation and cancer. 2 3 Most such compounds are metabolized by the mixed-function oxidase system located mainly in the endoplasmic reticulum.

The covalent binding of activated hydrocarbons to DNA has generated considerable interest. Quantitative measurements have also been performed on the binding of activated hydrocarbons to microsomal proteins. 4 No reports have appeared, however, on the specificity of metabolite binding to microsomal proteins, or whether there is a direct link between specific metabolite binding and cell toxicity.

To examine whether irreversible binding of metabolites to microsomal proteins occurs randomly or selectively, liver microsomes from control, phenobarbital and 3-methylcholanthrene (MC) pretreated rats were incubated with radiolabelled aromatic compounds in the presence of an NADPH-generating system. The binding patterns of the metabolites formed were obtained by sodium dodecylsulfate (SDS)-polyacrylamidegel electrophoresis and fluorography.

Experimental. Male Sprague-Dawley rats, weighing approximately 150 g, were maintained on a standard chow diet. Phenobarbital, 80 mg/kg, was injected intraperitoneally (i.p.) on three consecutive days and the animals were killed on the fourth day. MC, 80 mg/kg in corn oil, was injected i.p. 24 h before sacrifice. Protein was determined by the Lowry procedure 4 using bovine serum albumin as standard.

Microsomes were prepared as described earlier. 4 They were incubated with [14C]-phenol, [14C]-benzo[a]pyrene (BP) or [3H]-MC. The incubation mixture contained 0.6 ml of buffer (100 mM Tris-HCl, pH 7.5, 10 mM MgCl2 and 10 mM MnCl2), an NADPH-generating system (2 mg glucose-6-phosphate, 1.5 mg NADP and 2.7 \mu g (37 U) glucose-6-phosphate dehydro-

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genase), the radiolabelled compound dissolved in 10 \mu l of acetone (13 nmol [14C]-phenol, 35 mCi/mmol; 23 nmol [14C]-BP, 5 mCi/mmol; 0.92 nmol [3H]-MC 9 Ci/mmol) and 1.2—1.8 mg microsomal proteins in a total volume of 1.2 ml. The incubations were carried out at 37 °C for 60 min. When used, reduced glutathione (GSH) and UDP-glucuronic acid (UDPGA) were administered dissolved in 100 \mu l of water to give final concentrations of 2 and 15 mM, respectively.

The quantitative determination of irreversible binding to microsomal macromolecules was performed as described earlier, 4 except that the final protein pellet was dissolved in 0.5 ml Soluene-350 (Packard). A 0.2 ml aliquot of the protein solution was counted in 10 ml Dimiluene-30 (Packard).

Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis was performed as described in detail elsewhere. 5 8 The total monomer content of the gels was 11%, and 100—150 \mu g of microsomal protein was applied. The gels were treated with 2,5-diphenyloxazol (PPO) and dried * before fluorography. Kodak X-Omat films were exposed to the gels at —70 °C for 1—6 weeks. Molecular weight standards used were bovine serum albumin (Mw = 69 000), ovalbumin (Mr = 45 000), soybean trypsin inhibitor (Mr = 21 500), and sperm whale myoglobin (Mr = 17 200).

The electrophoretic binding patterns from control, phenobarbital and MC pretreated rats incubated with radiolabelled compounds are shown in Fig. 1. The binding pattern of phenol differs from those of BP and MC. The phenol pattern shows a dominant radioactive band with Mr = 72 000. Additional prominent bands are seen in the 50 000—60 000 Mr region. When microsomes from MC-induced animals were incubated, the bands in the 50 000—60 000 Mr region increased in intensity.

MC-induction affected the binding pattern of BP and MC metabolites much more than that of phenol metabolites. In MC-induced microsomes most of the binding occurred to at least three bands in the 60 000—60 000 Mr region, one band at Mr = 68 000 and one at Mr = 72 000 (this band is less prominent than the corresponding band in the phenol pattern). The 56 000 Mr band is seen after MC-induction only. It has the same mobility as cytochrome P-448, an enzyme which is specifically induced by MC. 5 6

The extent of irreversible metabolite binding to MC-induced microsomes was the highest for phenol. After 60 min of incubation, 2.8 nmol had bound per mg of membrane protein, corresponding to approximately 5% of the total amount of compound added. BP and MC metabolites bound to a much smaller extent, 0.8 and 0.6 nmol per mg membrane protein, corresponding to 1.5 and 1.2% of the added hydrocarbon.

The presence of GSH in the incubation medium prevented phenol metabolite binding
almost completely, while the binding of BP and MC metabolites was inhibited by approximately 65 %. UDPGA, which will conjugate with metabolites containing hydroxy groups, decreased the intensity of all bands in the BP and MC metabolite binding patterns. In order to bind the proteins, the toxic and/or carcinogenic compounds tested had to be metabolically activated, as indicated by the absolute requirement of an NADPH-generating system for binding.

Since the metabolites appeared to bind specifically rather than randomly to microsomal proteins, and since different protein binding patterns were observed for different compounds, the question arises which factors will determine the specificity of metabolite binding. One possibility is that metabolites will bind to any macromolecule with an exposed reactive group. This implies that any newly formed reactive metabolite most likely will bind to a reactive protein close to the site of metabolite formation. Thus, the enzyme that catalyses the formation of the metabolite would itself receive most of the binding provided it contains an exposed reactive group. An illustration of this point is the radioactive band of 56 000 $M_r$, which is obtained after incubation of all three compounds with MC-induced microsomes. This protein is probably cytochrome P-448, the enzyme thought to be responsible for most of the metabolic activation of polycyclic hydrocarbons in MC-induced microsomes.

Another possibility is that the kind of ultimate binding metabolite formed (for instance semiquinone or epoxide) determines the specific target protein(s). The ultimate binding metabolites for phenol are probably mainly quinones and semi-quinones. The main binding metabolites obtained from MC and BP have been suggested to be dihydrodiol epoxides and phenol epoxides.

The findings presented in this paper suggest that there are differences in binding patterns of metabolically activated compounds to microsomal proteins and that the patterns are dependent on the type of metabolite which is the ultimate binding agent.

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A Reaction Center Mutant of Rhodospirillum rubrum*

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Photosynthetic bacteria are especially suitable for a mutational analysis of the fundamental mechanisms of photosynthesis. Genetic techniques have, however, only recently been systematically introduced into the field of bacterial photosynthesis.1 Mutants of the photosynthetic bacterium Rhodospirillum rubrum, unable to grow photoheterotrophically, were isolated. This was accomplished by a method, whereby spontaneous mutants are enriched in the presence of 8.8 μg tetracyklin/ml under photoheterotrophic conditions (method suggested by B. Marrs, personal communication). One of the mutants obtained, B4, was grown semiaerobically to allow pigment synthesis, by gassing the medium continuously with 2.5 % O₂ and 5 % CO₂ in N₂. Chromatophore fragments were obtained from the mutant by procedures used for the wild type (strain S1).2 Absorption spectra of these chromatophore fragments showed the apparent absence of a peak at about 800 nm, which is present in wild type membranes (Fig. 1) and is due to a component in the reaction center called P800. Spontaneous revertants, which have regained the capacity to grow photosynthetically, can be isolated from B4 with a frequency indicating that the phenotype is caused by a single mutation. All the revertants isolated also regained P800. Among the revertants are, however, some with a significantly diminished content of P800.

Although the mutant chromatophores did not catalyze photophosphorylation and light-induced reduction of cytochrome b, energization of the membrane, measured as reduction of cytochrome b, was still possible in the dark with inorganic pyrophosphate. Light-induced energization returned in revertant chromatophores.

Analysis of the membrane fragments from B4 with SDS-polyacrylamide gel electrophoresis showed that a protein band corresponding to a molecular weight of about 18 000 dalton was lacking. This band reappeared in revertants with fully regained P800. In order to certify which one of the three different low molecular weight polypeptides in the reaction center that might be missing, it was necessary to isolate and analyze the reaction center complex from

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