Direct Formation of Complexes between Cytochrome P-450 and Nitrosoarenes†

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ABSTRACT: The mechanism of the formation of the complexes between various nitrosobenzenes and cytochrome P-450 has been investigated. We have observed the formation of these complexes by a new and, as yet, undescribed route. Nitrosobenzene (NOB) itself reacts with cytochrome P-450 in the iron(III) state, in the absence of any exogenous reducing agent, to produce the iron(II)–NOB complex. Apparently, NOB is a ligand that is capable of causing the spontaneous autoreduction of the iron. The reduction of the iron may occur via ligand-induced oxidation of the axially bound thiolate of cytochrome P-450.

The complexes formed by aliphatic and aromatic nitroso compounds with heme proteins such as cytochrome P-450 (P-450)[1] (Jonsson & Lindeke, 1976; Mansuy et al., 1977, 1978; Werringloer & Estabrook, 1973), myoglobin (Mansuy et al., 1978), or hemoglobin (Hirota et al., 1978) are of considerable interest because of their ability to alter the function of the heme protein. The nitroso function is isoelectronic with dioxygen and presumably binds to the heme in a manner analogous to it (Mansuy et al., 1983). The complexes formed with cytochrome P-450 are characterized by their absorption maxima at about 455 nm and are referred to as metabolic intermediate (MI) complexes (Franklin, 1977). Aromatic hydroxylamines form the same MI complex in the presence of Fe^{III} cytochrome P-450 (Fe^{III}-P-450). This is presumed to occur by a noncatalytic process in which the arylhydroxylamine is oxidized by Fe^{III}-P-450, producing the nitroso compound which then complexes to the resulting Fe^{II}-P-450 (Mansuy et al., 1978).

We have observed the formation of the MI complex by a new and surprising route. We found that nitrosobenzene (NOB) itself reacts with Fe^{III}-P-450 microsomes to produce the MI complex Fe^{II}-P-450–NOB in the absence of any detectable reducing agent. This result indicates that the NOB is a ligand capable of causing the autoreduction of the Fe^{III} heme, possibly by oxidation of the axial bound sulfide group. The reaction between *N*-hydroxyaniline (NOHA) and cytochrome P-450 was also reexamined because of the possibility that rapid oxidation of NOHA to NOB by air in aqueous buffer might have preceded formation of the MI complex observed in previous studies.

Materials and Methods

Materials. Nitrosobenzene was purchased from Aldrich Chemical Co. (Milwaukee, WI) and purified by multiple crystallizations from ether–ethanol. Other chemicals were purchased from commercial sources and used without further purification. Catalase was purchased from Boehringer Co. (Indianapolis, IN). Myoglobin (type III from horse heart) was obtained from Sigma Chemical Co. (St. Louis, MO).

Methods. Microsomes were prepared as previously described (Florence et al., 1982) from phenobarbital-pretreated male New Zealand rabbits (2–2.5 kg). Purified cytochrome P-450 LM_2 was isolated from phenobarbital-induced rabbit liver by procedures described earlier (Coon et al., 1978) to a specific activity of 10 nmol of P-450 LM_2/mg of protein and appeared as a single major band after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970). Protein content was determined by the method of Lowry (Lowry et al., 1951) with bovine serum albumin as standard.

Formation of the P-450–NOB complexes was measured by the method of Hlavica and Aichinger (1978). The microsomal incubation mixture contained approximately 2 μM (1 mg of protein/mL) cytochrome P-450, 0.15 M potassium phosphate buffer, pH 7.4, and 6 mM $MgCl_2$. The NADPH regenerating system, when used, was the same as that described (Hlavica & Aichinger, 1978).

The incubation mixture, without substrate was divided between two cuvettes. Substrate, dissolved in minimal acetonitrile or methanol, was added to a final concentration of 0.01–1 mM in the sample cell of a Cary 15 double-beam spectrophotometer. An equal volume of solvent was added to the reference cell such that the final concentration was 1%. The difference spectrum was recorded at 25 °C by scanning from 510 to 400 nm. Anaerobic solutions were obtained by nitrogen purging of all solutions. The samples were then sealed in cuvettes under positive nitrogen pressure.

Oxygen consumption experiments were performed with a YSI Model 53 biological oxygen monitor (Yellow Springs, OH) equipped with a Clark-type polarographic oxygen electrode.

Analysis for NOB was performed on an HPLC system consisting of an Altex Ultrasphere ODS column with a LKB Uvicord 2138 UV–vis detector (LKB-Produkter AB, Bromma, Sweden) operating at 206 nm. The eluant, MeOH–H_2O (75:25), was delivered with a minipump (Milton Roy Co., Riviera Beach, FL). Analysis for NOHA, azoxybenzene, and nitrobenzene, when required, was performed on a Hewlett-

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[1] Abbreviations: PB, phenobarbital; P-450, liver microsomal cytochrome P-450; NOB, nitrosobenzene; NOHA, *N*-hydroxyaniline; MI complex, metabolic intermediate complex; HPLC, high-pressure liquid chromatography; NADPH, reduced nicotinamide adenine dinucleotide phosphate; *A*, absorbance; LM_2, P-450 isozyme purified from PB-induced rabbit liver microsomes; TPP, tetraphenylporphyrin; ESR, electron spin resonance; THF, tetrahydrofuran; GC, gas chromatograph; MS, mass spectrum; CO, carbon monoxide; EDTA, ethylenediaminetetraacetate.

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Packard 5840 gas chromatograph with flame ionization detection and a 6′ OV 101 packed column. The samples were derivatized with trifluoroacetic anhydride overnight (for NOHA analysis) before injection into the GC. These compounds were characterized by coinjection with authentic samples and by GC/MS.

p-Chloro-, *p*-bromo-, *p*-methyl-, *p*-fluoro-, *p*-cyano-, and *p*-(trifluoromethyl)-*N*-hydroxyaniline were synthesized from the corresponding nitro compounds by the procedure described by Kamm (1941).

Iron(II) tetraphenylporphyrin (TPP) was prepared by stirring a toluene solution of FeTPPCl over zinc amalgam until the visible spectrum indicated that reduction was complete. This solution was then filtered through a 0.45-μm disposable filter and evaporated to dryness under vacuum. Visible spectra were recorded on a Beckman 5270 spectrophotometer with a 0.1-mm path-length cell. The $Fe^{III}TPPSbF_6$ was prepared as previously described (Quinn et al., 1982).

Toluene and tetrahydrofuran, used as solvents for the synthetic model porphyrins, were rigorously purified by distillation from sodium benzophenone under an argon atmosphere. All operations involving either the FeTPP or the $FeTPPSbF_6$ were carried out in a Vacuum Atmospheres dry box. The nitrosobenzene was dried in vacuo over P_2O_5 and then stored in the dry box.

ESR spectra were recorded on a IBM/Bruker ER200D-SRC spectrophotometer fitted with an Oxford Instruments cryostat for liquid helium use. The sample tubes were carefully flushed with He and sealed with epoxy cement to prevent condensation of air. The magnetic field was calibrated with an IBM/Bruker ER035M NMR gaussmeter and the frequency determined with an EIP Model 548A frequency counter. Spectra were typically recorded at 2.16 mW with a modulation amplitude of 5 × 1 and at a temperature of 9.0 K. In a typical ESR experiment, 200 μL of an incubation mixture containing 50 mg of microsomal protein/mL (100 μM P-450), 0.25 M phosphate buffer, pH 7.4, 20% glycerol, and 1 mM nitrosobenzene was monitored by the procedure of Nebert and Kon (1973).

The determination of the Fe^{II}–NOB complex formation from metmyoglobin (Mb^+) and from oxidized microsomes required an approximation of the extinction coefficient for each complex. Metmyoglobin was prepared by methods previously described (Hardman et al., 1966) and gave an absorbance maximum at 412 nm. The Mb^+ solution was deoxygenated under argon by successive purging and evacuation. An approximately 10-fold excess of sodium dithionite was added to obtain myoglobin (Mb). An approximately 50-fold excess of NOB in MeOH was added giving an absorbance maximum at 422 nm. Since the Mb^+ and Mb–NOB absolute spectra partially overlapped, a set of equations was developed to give the concentration for each species in a spectrum that is a composite of both [for example, see Castro et al. (1978)].

The extinction coefficient for the complex from microsomes was estimated as follows: NOB (1 mM) and microsomes (4.6 μM) were allowed to react until the difference between the absorbance at 453 nm and that of 500 nm was constant (approximately 50 min). Reduced CO difference spectra from both the sample and reference cuvettes were also obtained during this time. The reduced difference spectrum taken at a particular time did not change over a 5-min period. The apparent amount of cytochrome P-450 [obtained using an extinction coefficient of 91 mM^{-1} cm^{-1} (Omura & Sato, 1964)] in the sample cuvette decreased but became constant at 50 min, while that in the reference cuvette remained unchanged,

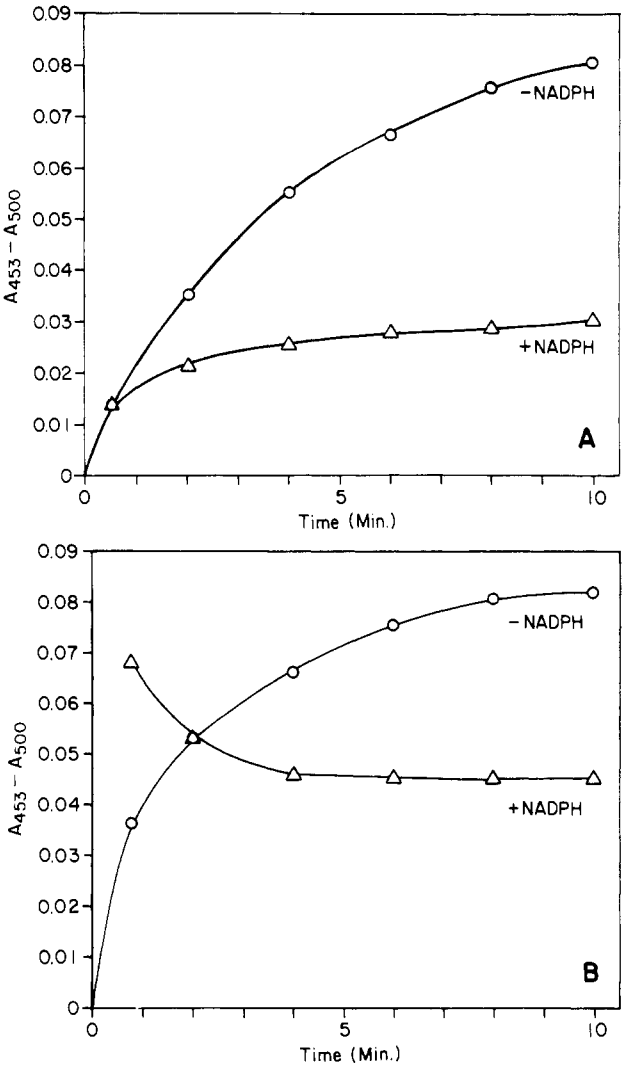


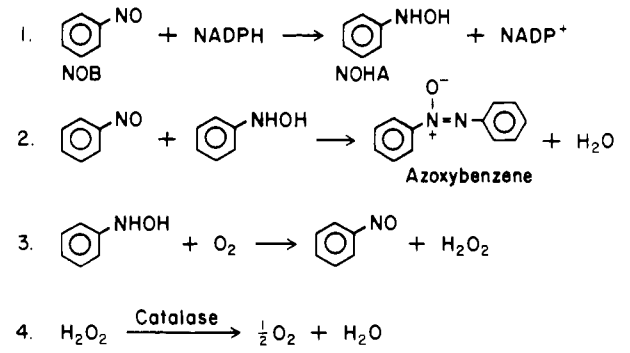
FIGURE 1: (A) Effect of NADPH on the rate of complex formation in aerobic microsomal solutions with added NOHA. (B) Effect of NADPH on the rate of complex formation in aerobic microsomal solutions with added NOB. The incubations contained 1.0 mM initial concentration of either NOHA or NOB, 3.5 μM P-450 and an NADPH-regenerating system as described under Methods.

indicating that the NOB blocked CO binding. This difference in apparent P-450 concentration gave an approximation of the amount of MI complex. Dividing the difference in the absorbance between 453 and 500 nm by this quantity gave a coefficient of 60 mM^{-1} cm^{-1}. Determination of the extinction coefficient by other methods (Franklin, 1974; Werringloer & Estabrook, 1975), which require incubation in the presence of known concentrations of ligand, was not possible in the present case since the aromatic nitroso compounds used dimerize to an unknown extent in aqueous solutions.

RESULTS

P-450 Complex Formation from NOB and NOHA. As expected from previous reports (Mansuy et al., 1978), reaction of NOHA with microsomes in the absence of NADPH caused the formation of the MI complex (Figure 1A), presumably by reduction of Fe^{III}-P-450 to the Fe^{II} protein, which then bound NOB. NOB is known to be formed in solution either by air oxidation of NOHA or as a result of the reaction between Fe^{III}-P-450 and NOHA. Unexpectedly, however, NOB itself was observed to cause the formation of the complex with microsomal (Figure 1B) or purified LM_2 (not shown) cytochrome P-450 in the absence of any added reducing agent. This observation was not expected since NOB is an unlikely

Chart I



reducing agent for the Fe^{III}-P-450 and only the Fe^{II} state should bind NOB.[2] The ability of NOB to form the complex directly with oxidized LM_2 indicated that other components of microsomes were not required.

In contrast, the complex between aliphatic *N*-hydroxylamines and cytochrome P-450 forms only in the presence of NADPH. This indicates that the alkylhydroxylamine is converted by cytochrome P-450 to the corresponding nitroso compound, which then binds to the NADPH-reduced enzyme (Mansuy et al., 1977b). The participation of an NADPH-dependent oxidative process for complex formation by the aromatic substrate *N*-hydroxyaniline was then tested. By analogy with the aliphatic systems, the rate of complex formation should have increased with the addition of NADPH, but the opposite effect is observed. The addition of NADPH to a microsomal suspension containing NOHA instead reduced the rate and extent of complex formation (Figure 1A). When NOB was added to a microsomal suspension that was pretreated with NAPDH, the complex formed quickly and then slowly decayed (Figure 1B), suggesting that NADPH can rapidly prereduce a portion of the cytochrome P-450 in the microsomal suspension, which then forms the complex with the added NOB. In support of this notion, carbon monoxide binding spectra determination (data not shown) indicated that 30% of the total microsomal P-450 could be reduced by NADPH alone. The nitroso ligand was also reductively removed by NADPH as evidenced by the gradual decay of the complex in the presence of NADPH (Figure 1B).

In order to determine the mechanism for the direct formation of the MI complex by NOB, a more thorough chemical investigation of substrate reactivity and complex formation was undertaken. The reactions of NOB and NOHA with both oxygen and NADPH were first investigated. Limiting factors in the study were the low solubility of NOB in aqueous media and its existence as an equilibrium mixture of monomer and dimer (Coombs, 1979). These properties of NOB precluded a direct quantitative comparison of its rate of complex formation with NOHA.

Reactivity of NOB and NOHA in Aqueous Buffer. NOB, in buffered solution, is stable to air oxidation but is readily and nonenzymatically reduced by NADPH (Becker & Sternson, 1980), which also appears able to reduce heme-bound NOB. Reduction of NOB by NADPH under aerobic conditions yielded azoxybenzene, its condensation product with NOHA, almost quantitatively. In contrast, anaerobic reduction substantially increased NOHA levels, indicating that the reduction of NOB by NADPH in the absence of oxygen was sufficiently rapid to compete favorably with azoxybenzene

[2] The addition of 2 equiv of the oxidizing agent $K_3Fe(CN)_6$ to the microsomal complex, formed in the presence of NOB, destroyed the complex.

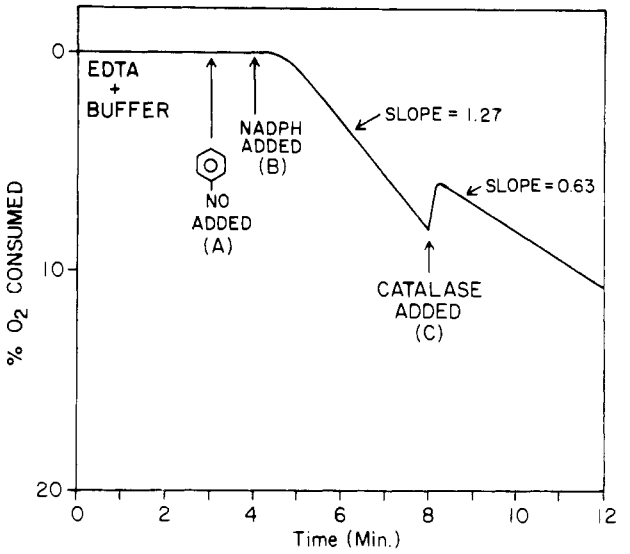


FIGURE 2: Oxygen consumption in a buffered solution (A) with NOB alone, (B) with NADPH plus NOB, and (C) with catalase (300 units) added. The conditions were similar to those of Figure 1B except that NADPH was added directly as the tetrasodium salt (to a final initial concentration of 1.3 mM), EDTA was present at 1 mM, and no microsomal protein was present.

formation. These reactions are summarized in Chart I, reactions 1 and 2.

Mansuy et al. (1978) have previously described the direct formation of the Fe^{II}–NOB complex but considered it to be a consequence of the presence of NOHA formed by disproportionation of NOB since they had also shown that NOHA rapidly forms the complex. However, the rapid aerobic oxidation of NOHA to NOB does not appear to have been considered. When buffered solutions of NOHA were monitored with an oxygen-sensitive electrode, rapid consumption of oxygen was noted with formation of NOB and H_2O_2. NOB was determined by HPLC procedures, and H_2O_2 was determined by monitoring changes in O_2 levels due to added catalase. Thus, when a solution of NOHA, generated by reduction of NOB with NADPH, was allowed to stand, it rapidly consumed oxygen. If oxygen were removed from this solution by bubbling it with nitrogen, oxygen evolution resulting from catalase action was noted. Alternatively, if catalase were present during the oxidation process, the rate of oxygen consumption was decreased by exactly half as predicted by the stoichiometry of the catalase reaction (Chart I, reactions 1, 3, and 4, and Figure 2).

Effect of Oxygen on Rate of Complex Formation. Part of the decrease observed in the rate of complex formation between NOHA and Fe^{III}-P-450 and between NOB and Fe^{III}-P-450 when NADPH was present in the incubation mixture can be accounted for by changes in NOB concentration. NOB apparently forms the complex with Fe^{III}-P-450 more rapidly than does NOHA and is generated rapidly from NOHA by air oxidation. When NADPH is present, however, it causes the conversion of NOB to NOHA so that the overall effect is to decrease the concentration of NOB. Consistent with this notion is the observation that the rate of complex formation between NOHA and Fe^{III}-P-450 in the absence of either air or NADPH was dramatically increased upon opening the vessel to air (Figure 3), allowing oxidation of NOHA to NOB. By contrast, removal of oxygen from solution had little or no effect on complex formation from NOB and Fe^{III}-P-450. All of the microsomal suspensions were shown to be free of Fe^{II}-P-450 by direct CO binding analysis prior to the addition of substrate.

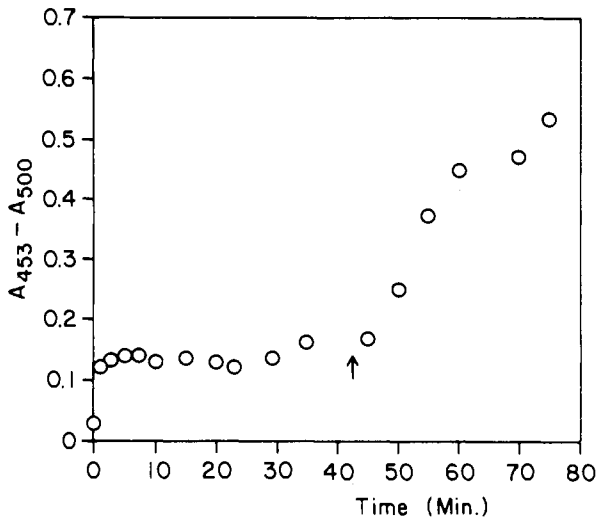


FIGURE 3: Effect of oxygen on the rate of complex formation in a microsomal incubation in the presence of 1.0 mM NOHA. The incubation conditions were the same as described in Figure 1A, in the absence of NADPH, except that oxygen was excluded as described under Methods. The cuvette was opened to the air at the time indicated by the arrow.

Since NOHA can reduce Fe^{III}-P-450, it is possible that, in the presence of oxygen, NOHA could support the catalytic conversion of NOHA to NOB by P-450. This could be an alternative explanation for the effect of oxygen on the rate of complex formation; i.e., catalytic aerobic oxidation of NOHA to NOB by P-450 with concomitant Fe^{III} reduction would lead to the complex. To eliminate the possibility that NOHA was replacing NADPH as the electron source in the P-450 catalytic cycle, the demethylation of benzphetamine was monitored in suspensions of microsomes containing only NOHA as the electron source. Although complex formation would be inhibitory and reduce the catalytic activity of the microsomes, some demethylation would be expected. None was observed.

In further support of the concept that direct NOB binding to P-450 is responsible for a large portion of the MI complex, a positive correlation between the relative rates of air oxidation of various para-substituted *N*-hydroxyanilines and their relative rates of complex formation in aerobic incubations was obtained (Figure 4). Thus, the rate of formation of a large portion of the MI complex between aromatic hydroxylamines and P-450 depends on air oxidation of NOHA to NOB.

It is noteworthy that a number of the interactions of aromatic nitroso compounds are not observed with aliphatic hydroxylamines or nitroso compounds. For example, *N*-hydroxyamphetamine will only form the MI complex in the presence of NADPH (Kawalek et al., 1976). Aliphatic hydroxylamines are also very slowly oxidized by oxygen, and the corresponding nitroso compound is not reduced by NADPH at a measurable rate.

Possible Disproportionation of NOB in Aqueous Buffer. As stated earlier, the direct formation of the complex by NOB has been observed previously and was explained on the basis of a predisproportionation of NOB to generate NOHA (Mansuy et al., 1978). A direct study of the possible disproportionation of NOB was therefore performed. Neither nitrobenzene nor azoxybenzene[3] was detected in incubation mixtures of NOB and microsomes after extraction and GC analysis by procedures of sufficient sensitivity to detect disproportionation products expected from the levels of complex formed. Direct HPLC analysis of the incubation mixture was also unable to detect nitrobenzene or azoxybenzene. Finally, the absorption at 308 and 281 nm, characteristic of nitrobenzene (Becker & Sternson, 1980), was unchanged over a 30-min period when a buffered 0.1 mM solution of NOB was incubated under conditions equivalent to the microsomal incubation. Thus, under the reaction conditions and time span required for complex formation, NOB is stable in the presence and absence of microsomes and does not disproportionate.

[3] The disproportionation of NOB will presumably generate nitrobenzene and NOHA. Also, azoxybenzene should be seen from the condensation of NOHA with unreacted NOB.

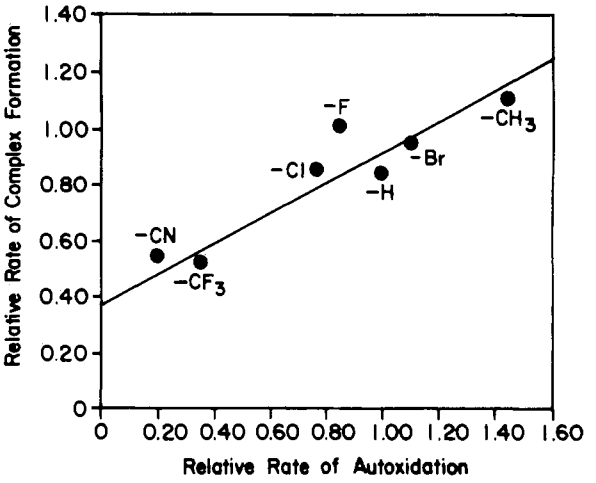


FIGURE 4: Correlation of the relative initial rates of complex formation with the relative rates of air oxidation for a series of para-substituted *N*-hydroxyamines. Conditions were the same as those in Figures 1A (in the absence of NADPH) and 2.

Properties of a Model Synthetic Porphyrin System. These data raised the possibility that NOB was somehow binding directly to Fe^{III}-P-450 by a mechanism that had not yet been considered. Although the nitroso function is expected only to complex with Fe^{II}-P-450, the possibility that it could complex with an Fe^{III} heme was examined with a model synthetic porphyrin system. The addition of excess NOB to (tetraphenylporphyrinato)iron(III) hexafluoroantimonate, Fe^{III}TPPSbF_6, in tetrahydrofuran (THF) or in toluene under anaerobic conditions had no effect on the original spectrum of the Fe^{III} salt. This particular Fe^{III} porphyrin is extremely susceptible to ligand binding since the SbF_6 counterion is easily displaced by any Lewis base (Quinn et al., 1982). The lack of binding of NOB with this iron porphyrin indicates that, as expected, NOB has a very low binding constant with Fe^{III} porphyrins. On the other hand, when NOB was added to (tetraphenylporphyrinato)iron(II), Fe^{II}TPP, in THF, a spectral shift occurred in the visible spectrum indicating complexation (Figure 5a). When NOB was added to a solution of Fe^{III}TPPCl, there were no spectral changes, but the addition of thiophenoxide to the mixture generated the same visible spectrum as that seen with NOB and Fe^{II}TPP (Figure 5b). Thus, the thiolate appears to be capable of reducing Fe^{III} to Fe^{II}, which then binds NOB. An alternative explanation would be that the thiolate reduces NOB to the NOB radical anion (Smentowski, 1963), which then binds to the Fe^{III}-state porphyrin. However, since the axial ligand of P-450 is known to be a thiolate function, these experiments suggest that it can act as an endogenous reducing agent that reduces Fe^{III}-P-450 for binding by NOB.

Effect of NOB on Microsomal Electron Paramagnetic Resonance Spectrum. If the binding of NOB to the active site of cytochrome P-450 induces a one-electron reduction of Fe^{III}, the change in electron distribution should be discernible by ESR techniques. Preliminary studies indicate that the

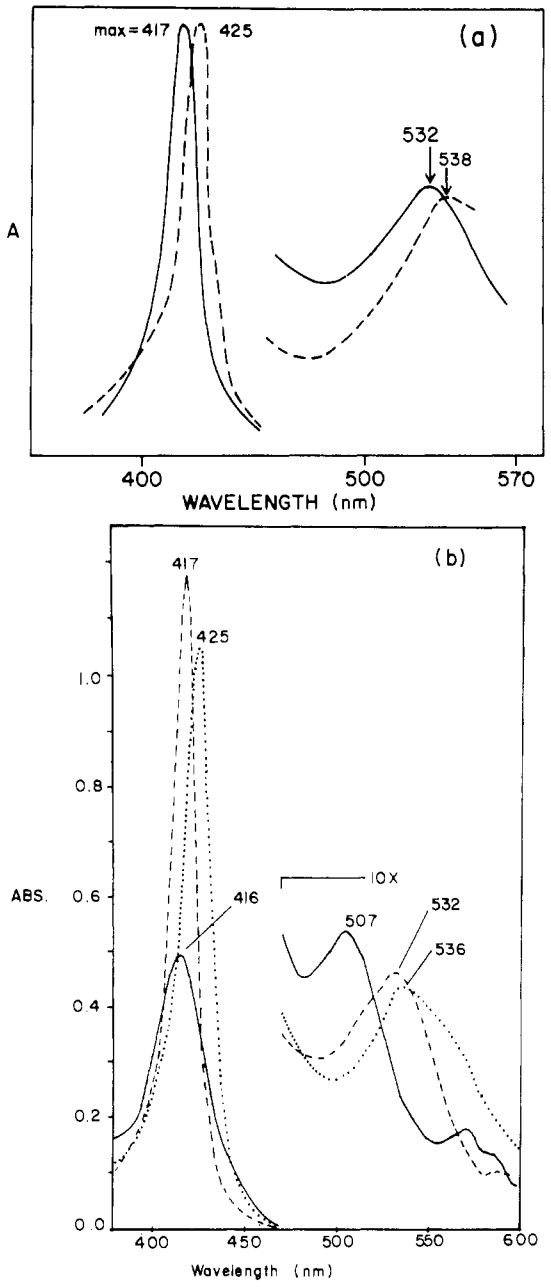


FIGURE 5: (a) Visible spectra of model porphyrin systems: (--) $Fe^{II}TPP$; (—) $Fe^{II}TPP$ with added NOB. (b) Visible spectra of the model porphyrin systems: (—) $Fe^{III}TPPCl$; (···) $Fe^{III}TPPCl$ plus benzene thiolate; (--) $Fe^{III}TPPCl$ plus NOB plus benzene thiolate. All spectra were recorded in dry, degassed THF.

addition of a concentrated NOB solution in acetonitrile to a microsomal preparation in the absence of any reducing agent dramatically reduced the high- and low-spin Fe^{III} signals (Figure 6). This change would result if the Fe^{III} were converted to the ESR-silent Fe^{II} state by NOB binding. If an axial thiol was acting as a reducing agent, a thiyl radical would form that cannot normally be detected by ESR (Symons, 1974; Gilbert et al., 1975).

Comparison of Complex Forming Ability of Myoglobin vs. Cytochrome P-450. The hemoprotein myoglobin is also capable of forming an NOB–myoglobin complex (Mansuy et al., 1978). A comparison of the direct NOB binding was then made between Fe^{III} myoglobin (metmyoglobin) and Fe^{III}-P-450. Under identical conditions, i.e., heme concentration, NOB concentration, buffer, etc., NOB was found to directly bind microsomal Fe^{III}-P-450 approximately 6 times faster than Fe^{III} myoglobin. This, presumably, is a conservative estimate

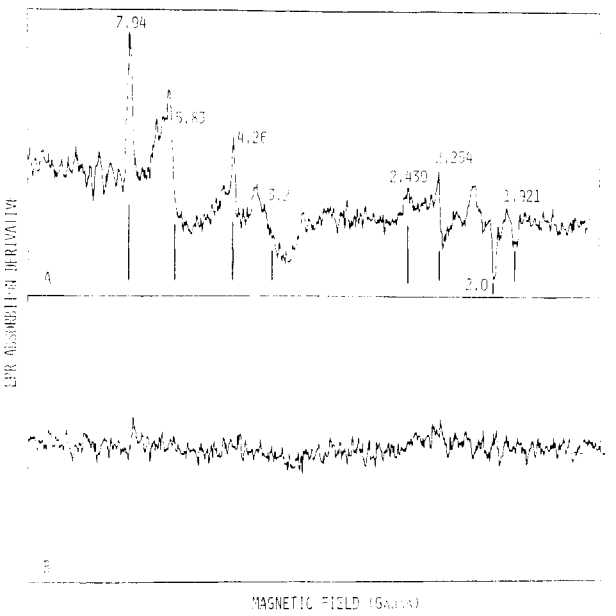


FIGURE 6: (A) ESR spectrum of a microsomal suspension containing 1% acetonitrile. (B) ESR spectrum of microsomes incubated for 20 min in the presence of 1 mM NOB and 1% acetonitrile. The conditions for the ESR experiments are described under Methods. The ESR spectra were recorded at 9.0 K at 9.4350 GHz. The g values of the various signals are noted on the spectrum. The signal at $g = 2.0$ is probably due to an extraneous radical.

since the NOB complex will probably not form with all the isozymes present in a microsomal suspension and yet all the isozymes are included in the value for the total P-450 concentration. Thus, there appears to be something unique about cytochrome P-450 that allows it to be autoreduced at a significantly faster rate compared to metmyoglobin.

Discussion

The evidence presented indicates that MI complexes can form by direct interaction between NOB and Fe^{III} and that the previously proposed mechanism by which NOHA forms these complexes appears to be only partially responsible for the total amount formed in aerobic NOHA–microsomal mixtures. The decrease in the rate of complex formation with the addition of NADPH to microsomal suspensions containing NOHA can be accounted for by the decrease in levels of NOB, generated from the air oxidation of NOHA, and the assumption that NOB is responsible for most of the complex formation. In support of this hypothesis, we and others (Becker & Sternson, 1980) have shown that NADPH will reduce NOB to NOHA. Furthermore, the spontaneous air oxidation of NOHA to NOB and hydrogen peroxide in buffer solutions was shown to be sufficiently rapid for the time span needed for complex formation.

NOHA only very slowly forms the MI complex with microsomal P-450 under anaerobic conditions, but complex formation rapidly increases when air is allowed into the reaction vessel (Figure 3), presumably as a result of NOB formation. In another series of experiments, the rate of oxidation of a series of para-substituted *N*-hydroxyanilines and their respective rates of complex formation were found to correlate such that the faster the rate of air oxidation, the faster the rate of complex formation (Figure 4), again suggesting that air oxidation of NOHA is a determining factor in the overall rate of complex formation.

The rapid generation of the complex after addition of pure NOB to microsomal suspensions has been observed previously and rationalized on the basis of a predisproportionation of

NOB to generate NOHA, which reduces Fe^{III} to allow binding with NOB (Mansuy et al., 1978). However, we were unable to detect any disproportionation products in these studies and therefore believe that NOHA formation is not a prerequisite for complexing with Fe^{III}-P-450 but that NOB alone will form the MI complex in the absence of exogenous reducing agent. In further support of this notion, the addition of NOB to a microsomal suspension resulted in a net decrease in detectable ESR signals. Both the high- and low-spin forms of Fe^{III} are reduced with NOB, indicating a net change of the oxidation state of the heme iron to Fe^{II}. Experiments with model porphyrin systems indicated that NOB will only bind Fe^{II} porphyrins. The apparent inability of NOB to bind to Fe^{III}-P-450, together with the ESR results, indicates that NOB binds cytochrome P-450 and spontaneously causes the autoreduction of Fe^{III}-P-450 to Fe^{II}-P-450. Ligand-induced autoreduction of Fe^{III} porphyrins to the Fe^{II} porphyrins is a well-documented phenomenon with such ligands as pyridine or cyanide ion (La Mar & Del Gaudio, 1977). The addition of phenyl thiolate to a Fe^{III}TPPCl–NOB solution results in the identical spectrum obtained when Fe^{II}TPPCl and NOB are mixed. Although the initial finding is not yet accountable in terms of a specific mechanism, this observation indicates that the thermodynamics of the reduction of Fe^{III} porphyrins by thiolates is favorable.

The reduction of Fe^{III} salts to Fe^{II} salts by NOHA has been shown to occur (Mullvey & Waters, 1977) and is probably responsible for part of the prereduction of Fe^{III}-P-450 prior to NOB binding in aerobic NOHA solutions. Since it has been demonstrated that NOB will only bind Fe^{II} hemes and since NOB is incapable of reducing the oxidized heme, it remains to be determined how the heme is reduced. One possibility, though speculative, is that the combination of the binding affinity of NOB to the hydrophobic active site of P-450 combined with the very high affinity of NOB for Fe^{II} porphyrins makes the internal reduction of the Fe^{III} porphyrin by its axial thiolate ligand thermodynamically favorable. Whether the resulting thiol radical is, in turn, reduced by some other group in the protein is, as yet, unknown. Essentially, one can look at the iron as a conduit for the reduction of the NOB ligand by thiolate, a process known to occur without the metal ion (Smentowski, 1963).

Consistent with the notion of NOB-initiated autoreduction of an axially bound thiolate group on the heme is the finding that metmyoglobin (Mb^+), another Fe^{III} protein, is significantly slower in forming the corresponding complex than cytochrome P-450. Myoglobin–NOB complexes are known and have been characterized previously (Mansuy et al., 1978). In contrast to cytochrome P-450, myoglobin does not have an easily oxidizable axial ligand and thus is not as likely to autoreduce in the presence of NOB. This, however, is not a rigorous argument due to the structural dissimilarities between myoglobin and cytochrome P-450, but it is consistent with the mechanistic scheme presented here.

Summary. Our data indicate that NOB reacts directly with Fe^{III}-P-450 to produce the MI complex Fe^{II}-P-450–NOB. The source of the reducing equivalent needed to reduce Fe^{III} to Fe^{II} is as yet unknown, but it is possible that it is the axial thiolate ligand bound to Fe^{III} in P-450. Alternatively, some other endogenous reducing factor could be present in the protein that could reduce the iron to allow NOB binding.

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