Oxidation of Substituted *N,N*-Dimethylanilines by Cytochrome P-450: Estimation of the Effective Oxidation–Reduction Potential of Cytochrome P-450†

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ABSTRACT: Rates of N-demethylation of *N,N*-dimethylaniline and of eight meta- or para-substituted *N,N*-dimethylanilines by rat liver cytochrome P-450$_{PB-B}$ (P-450) were determined under conditions where oxidation was supported by iodosylbenzene or NADPH–P-450 reductase. The rates of dimethylaniline oxidation were found to correlate with the substrate oxidation–reduction potential within each series of substrates supported by a particular oxygen activation protocol; the k_{cat} for each substrate studied was approximately 20-fold faster in the iodosylbenzene-supported system relative to the NADPH–P-450 reductase supported system. Since the N-demethylation of amines is believed to proceed via an initial electron-transfer step, a kinetic scheme for P-450 was proposed that enabled evaluation of the data according to theoretical treatments that correlate rates of electron transfer with extrakinetic parameters. In these analyses, the data could be fitted to the Rehm–Weller and Agmon–Levine equations, providing λ values (for the energetics of enzyme–substrate reorganization) of 22–26 kcal mol^{-1} and apparent $E_{1/2}$ (oxidation–reduction potentials) of 1.7–2.0 V (vs saturated calomel) for the oxidized enzyme. The apparent $E_{1/2}$ for the enzyme is composed of contributions from the intrinsic potential of the active prosthetic core of the enzyme, the Fe=O—porphyrin species, and a coulombic factor that is a function of the charge-separated radical anion/radical cation pair produced upon electron transfer. The electrostatic contribution to the enhancement of the enzyme $E_{1/2(app)}$ is enabled by the charge donation afforded the Fe=O species by the axial thiolate species and is proposed to be significant (<~0.85 V), as a consequence of the low dielectric constant predicted for the enzyme active site.

Cytochrome P-450[1] enzymes are primarily involved in mixed-function oxidation reactions; the different P-450s collectively oxidize a great variety of organic substrates (Guengerich, 1987). The mechanism by which triplet oxygen is activated to a reactive form and added to a diverse range of substrates has been the focus of considerable attention. Figure 1 depicts a commonly accepted working scheme for catalysis (White & Coon, 1980; Guengerich & Macdonald, 1984; Ortiz de Montellano, 1986). This paper will be restricted to the analysis of the portion of the scheme depicted by P-450(S) $(FeO)^{3+}$ to P-450 $(SO)(Fe^{3+})$, in which an atom of oxygen is transferred from the heme iron to the substrate. Two pathways can be used to reach the putative reactive $(FeO)^{3+}$ species, one involving NADPH–P-450 reductase supported reduction in the presence of O_2 and the other involving artificial oxygen atom donors such as iodosylbenzene.

The chemical mechanism of P-450 oxidations can be viewed in a unified scheme in which the $(FeO)^{3+}$ species abstracts a hydrogen atom or a nonbonded or π electron in the first step; the oxygenation reaction is then completed by radical recombination (oxygen rebound) (Guengerich & Macdonald, 1984; McMurry & Groves, 1986; Ortiz de Montellano, 1986). One of the more thoroughly studied P-450 reactions is that of amine N-dealkylation, which is generally accepted to proceed via initial electron transfer followed by rapid deprotonation of the nitrogen-centered radical cation intermediate and subsequent collapse (Figure 2). Support for this view comes from several lines of evidence. (1) *N*-Oxides are not rearranged to carbinolamines by P-450 (Guengerich, 1984) and, indeed, hydroxylamines (Kadlubar & Hammons, 1987) and *N*-oxides (Guengerich et al., 1986) are formed when α-hydrogens are inaccessible (Guengerich & Macdonald, 1984). (2) Cyclopropylamines (and other cyclopropyl-substituted heteroatoms) are oxidized by P-450, and the reaction products are indicative of stepwise electron-transfer processes (Macdonald et al., 1982; Hanzlik & Tullman, 1982; Guengerich et al., 1984). (3) Low intrinsic kinetic deuterium isotope effects have been interpreted as being consistent with such a mechanism (Shea et al., 1983; Miwa et al., 1983). (4) The oxidation of 1,4-dihydropyridines by P-450 occurs with lack of isotopic and stereochemical discrimination at C-4 (Guengerich & Böcker, 1988) and release of 4-alkyl substituents as radicals (Augusto et al., 1982). (5) Hammett analysis of rates of N-demethylation of a series of substituted *N,N*-dimethylanilines by P-450 yields a negative ρ value, interpreted in terms of a positively charged intermediate (Burka et al., 1985; Galliani et al., 1984). Thus, considerable precedent exists for electron transfer as the initial chemical step in the oxidation of alkylamines by the P-450 active oxygen species. We have extended our previous ob-

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[1] Abbreviations: P-450, cytochrome P-450; P-450$_{PB-B}$, a major form of liver microsomal P-450 purified from phenobarbital-induced rats; $E_{1/2(app)}$, effective oxidation–reduction potential of cytochrome P-450 (formal FeO^{3+} complex) or other catalyst defined relative to SCE; $E_{1/2(subs)}$, oxidation–reduction potential of substrate; $E_{1/2(int)}$, the intrinsic oxidation–reduction potential devoid of electrostatic contributions; $E_{(cf)}$, coulombic factor derived from electrostatic interactions in the transition state for electron transfer; SCE, saturated calomel electrode.

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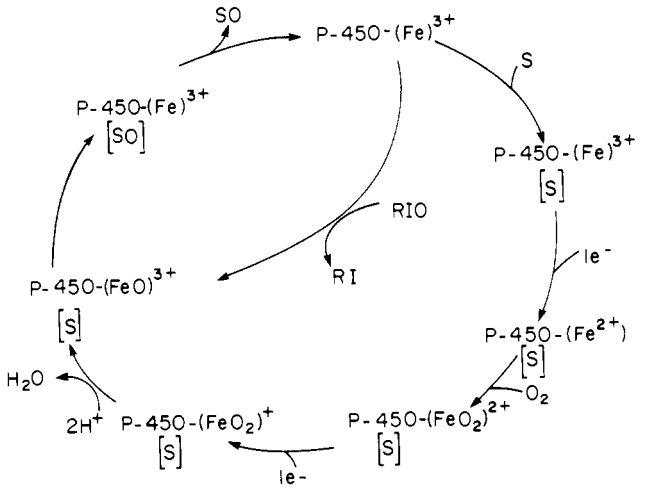

FIGURE 1: P-450 catalytic cycle.

servations (Burka et al., 1985) concerning the relationship between k_{cat} for oxidation and the electronic parameters of the substrate to obtain an estimate of the oxidation potential of the P-450.

The electron-transfer chemistry in P-450 reactions must be a function of parameters of the active oxygen species depicted in Figures 1 and 2, $(FeO)^{3+}$, particularly the $E_{1/2(app)}$. However, the electron-transfer processes of this activated intermediate are obscured by other steps in the catalytic cycle. For example, oxidation rates are often found to be much higher when the NADPH–P-450 reductase mediated pathway of oxygen activation is replaced by oxygen transfer from artificial oxygen donors (RIO in Figure 1) (McMurry & Groves, 1986). Formal $(FeO)^{3+}$ species in model metalloporphyrins have been generated electrochemically, and their oxidation potentials have been measured (Lee et al., 1985; Groves & Gilbert, 1986). However, these intermediates are short-lived and cannot be measured directly in P-450 proteins; thus, indirect approaches for the determination of the oxidation potential are necessary. One approach involves the analysis of relationships between kinetic and nonkinetic data. It has now been established that an inverse relationship between rates of S-oxygenation and $E_{1/2(app)}$ exists for thioanisole S-oxygenation with lactoperoxidase, chloroperoxidase, and horseradish peroxidase (Doerge, 1986; Kobayashi et al., 1987). Further, crude and purified P-450 preparations show similar relationships for thioanisole S-oxygenation (Watanabe et al., 1982) and *N*,*N*-dimethylaniline *N*-demethylation (Galliani et al., 1984, 1986; Burka et al., 1985). While these data have all been considered in terms of Hammett analysis, the approach is limited in the amount of mechanistic understanding that can be gained.

Both theoretical and empirical approaches have been developed to investigate the relationship between rates of enzyme catalysis and $E_{1/2(subs)}$ differences among substrates. Some biological applications have been described by Marcus and Sutin (1985) and by Cusanovich and Meyer (Meyer et al., 1983, 1986; Przysiecki et al., 1985). To date the applications of rate theories in biological settings have focused on evaluation of electron transfer among well-characterized redox partners. We have attempted for some time to gain insight into the effective oxidation potential ($E_{1/2(app)}$ of the transient active intermediate of P-450 oxidation. The determination of the effective oxidation potential of the intact enzyme is confounded by the complex nature of the catalytic cycle of P-450 (Figure 1) and by the fact that the intermediate of interest (the Fe=O^{3+} species) is "buried" at the center of the catalytic mechanism. In this paper, we extend an approach developed

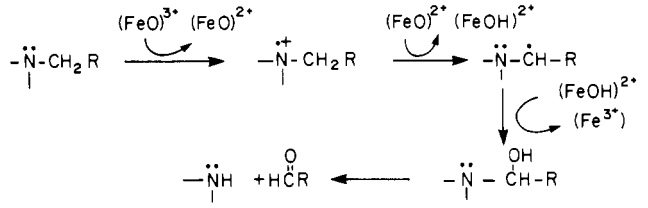

FIGURE 2: Proposed mechanism of N-demethylation by P-450.

to determine the oxidation potentials of transient excited-state species (Ballardini et al., 1978; Bock et al., 1979) to a biological system, P-450-catalyzed alkylamine N-demethylation. Assuming that the chemical mechanism of amine oxidation by P-450 involves initial single-electron transfer from substrate to enzyme, we have employed this approach to characterize the relationship between the rates of substrate oxidation and the oxidation-reduction potential difference between the P-450 enzyme and its substrates. This approach provides the first estimate of the effective oxidation–reduction potential for the P-450 iron–oxo species in the intact enzyme.

Experimental Procedures

Enzymes. P-450 (P-450$_{PB-B}$) and NADPH–P-450 reductase were purified from liver microsomes of phenobarbital-treated rats by using minor modifications of techniques described elsewhere (Guengerich & Martin, 1980; Guengerich et al., 1982; Yasukochi & Masters, 1976) and were homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Electrochemistry. The $E_{1/2(subs)}$ values of all substrates were determined by cyclic voltammetry using a Princeton Applied Research Model 173 potentiostat and 174A polographic analyzer with an EG&G Model 175 universal programmer employing a platinum working electrode and a Ag/AgCl (1.0 M) reference electrode. Solutions of the substituted dimethylanilines were studied at 1.0 mM concentrations in anhydrous CH_3CN containing 0.1 M tetra-*n*-butylammonium perchlorate purged with nitrogen. The scan rate was 500 mV s^{-1}. $E_{1/2(subs)}$ values for the dimethylaniline substrates were compiled in Table I (corrected to SCE). The values for 4-CO_2CH_3, 4-NO_2-, 4-CN-, and 4-Br-*N*,*N*-$(CH_3)_2$-aniline were quasi-reversible; the remaining $E_{1/2(subs)}$ values were reversible.

Chemicals. Para-substituted *N*,*N*-dimethylanilines were purchased from Aldrich Chemical Co., Milwaukee, WI (4-Br, 4-CN, 4-CH_3, 3-CH_3, 4-CHO, 4-H), CTC Organics, Atlanta, GA (4-F), and Chem Service, Inc., West Chester, PA (4-Cl) or the anilines were purchased and methylated as described by Gribble and Nutaitis (1985) (4-CO_2CH_3 from Aldrich) or with dimethyl sulfate (4-NO_2 from Aldrich). Each dimethylaniline was dissolved in water (HCl salt, 45 mM) by sonication.

Enzyme Assays and Analysis of Data. Two different approaches were used for the P-450-dependent systems.

In NADPH–P-450 reductase supported reactions, 0.3 nmol of P-450$_{PB-B}$, 0.42 nmol of NADPH–P-450 reductase, and 4.5 nmol of L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine were mixed together. After 5 min at 23 °C, the volume was made up to 0.125 mL, with a final concentration of 50 mM potassium phosphate buffer (pH 7.7) and a 0.02–2 mM concentration of the *N*,*N*-dimethylaniline under consideration. The anilines were added as their hydrochloride salts (aqueous solutions). Mixtures were incubated at 37 °C for 3 min, and then reactions were initiated by the addition of 25 μL of an NADPH-generating system containing 67 mM glucose 6-phosphate, 6.7 mM $NADP^+$, and 0.67 μg of yeast glucose-

Table I: Substituted *N,N*-Dimethylaniline Oxidation Data and $E_{1/2(subs)}$ Data for the NADPH–P-450 Reductase Supported and Iodosylbenzene-Supported Oxidation Systems

N,N-dimethylaniline substituent	$E_{1/2(subs)}$ [V (SCE)]	oxidation system			
		NADPH–P-450 reductase		iodosylbenzene	
		k_{cat}[a] (min^{-1})	K_m[a] (mM)	k_{cat}[a] (min^{-1})	K_m[a] (mM)
3-CH_3	0.74	14.7 (±0.5)	0.15 (±0.02)	232 (±10)	0.96 (±0.06)
4-CH_3	0.75	12.7 (±0.3)	0.16 (±0.02)		
4-H	0.80	10.6 (±0.4)	0.26 (±0.03)	182 (±12)	0.76 (±0.13)
4-F	0.87	9.2 (±0.2)	0.08 (±0.01)		
4-Cl	0.92	9.0 (±0.4)	0.07 (±0.01)		
4-Br	0.96	7.5 (±0.2)	0.042 (±0.003)		
4-CHO	1.14	4.3 (±0.3)	0.37 (±0.08)	82 (±8)	0.26 (±0.06)
4-CN	1.18	2.7 (±0.4)	0.07 (±0.03)	77 (±9)	0.67 (±0.09)
4-NO_2	1.27	1.4 (±0.2)	0.03 (±0.01)	47 (±8)	0.04 (±0.02)

[a] Numbers in parentheses represent one standard deviation.

Table II: Summary of the Analysis of the NADPH–P-450 Reductase Supported and Iodosylbenzene-Supported Demethylation Data by Rehm–Weller and Agmon–Levine Approaches

	A[a] (min^{-1})	λ[a] (kcal mol^{-1})	$E_{1/2(app)}$[a] [V (SCE)]
NADPH–P-450 Reductase Supported Systems			
Rehm–Weller	130 (±30)	22.6 (±1.6)	1.70 (±0.06)
Argmon–Levine	24 (±3)	21.8 (±1.0)	1.78 (±0.04)
Iodosylbenzene-Supported Systems			
Rehm–Weller	2400 (±1200)	25.8 (±3.1)	1.96 (±0.09)
Agmon–Levine	370 (±40)	23.9 (±0.6)	1.97 (±0.05)

[a] Numbers in parentheses represent one standard deviation.

6-phosphate dehydrogenase per milliliter. Incubations proceeded for 5 min at 37 °C and were quenched by the addition of 50 μL of 17% $HClO_4$; HCHO was estimated colorimetrically as described elsewhere (Nash, 1953; Cochin & Axelrod, 1959).

The procedure was similar in the cases where the reaction was supported by iodosylbenzene. Each incubate contained 0.66 nmol of $P\text{-}450_{PB\text{-}B}$ and 4.5 nmol of L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine; NADPH–P-450 reductase was not added, but phosphate buffer and the substrate (substituted *N,N*-dimethylaniline) were added as described above for the NADPH–P-450 reductase supported reactions. Mixtures were preincubated for 3 min at 37 °C, and then iodosylbenzene was added to a final concentration of 2 mM with mixing on a vortex device (from an aqueous stock solution of 10 mM, which had been dissolved by sonication). After 30 s (a time point at which reactions were still apparently linear with respect to product formation vs time), reactions were quenched with $HClO_4$ as above and HCHO was measured. In these experiments, the mixtures were not heated in the course of the Nash (HCHO assay); control incubations were carried out at each substrate concentration that was devoid of P-450 (corrections were also made for any color observed in incubations containing only P-450 and iodosylbenzene).

Rate parameters (V_{max}, K_m) were derived from nonlinear least-squares fitting to the standard enzyme–substrate (Michaelis–Menten) kinetics ($v = V_{max}[S]/[S] + K_m$); k_{cat} was derived by dividing V_{max} by the enzyme concentration.

The rates obtained for each substrate investigated by these oxygen-activated protocols are compiled in Table I. The data were analyzed in the following manner. The k_{cat} values of Table I were fitted to the Marcus, Rehm–Weller, and Agmon–Levine equations for electron transfer [see: Eberson (1981), Meyer et al. (1983), and Murdoch (1983)]. Nonlinear least-squares analysis was performed with the best fitting of the most error-prone observable, k_{cat}, according to

$$k_{cat} = Ae^{-(\Delta G^+/RT)} \quad (1)$$

where T is the absolute temperature, R is the gas constant, A is a preexponential factor, and ΔG^+ is the free energy of activation for electron transfer within the association complex.

The free energy of activation takes different expressions in the three treatments:

Marcus

$$\Delta G^+ = \frac{\lambda}{4}\left(1 + \frac{\Delta G^\circ}{\lambda}\right)^2 \quad (2)$$

Rehm–Weller

$$\Delta G^+ = \Delta G^\circ/2 + [(\Delta G^\circ/2)^2 + (\lambda/4)^2]^{1/2} \quad (3)$$

Agmon–Levine

$$\Delta G^+ = \Delta G^\circ + \frac{\lambda}{4 \ln 2} \ln [1 + e^{-4\ln 2(\Delta G^\circ/\lambda)}] \quad (4)$$

where ΔG° is the standard free energy change for electron transfer and λ is the reorganization energy.

The relationship between the maximal rate of substrate oxidation (k_{cat}) and the oxidation potential of the substrate ($E_{1/2(subs)}$) was analyzed according to eq 1, in which the free energy of activation is evaluated by three rate-equilibria treatments of electron transfer. All of these extrakinetic expressions for assessment of electron transfer, the Marcus (eq 2), Rehm–Weller (eq 3), and Agmon–Levine (eq 4) equations, have been shown to be special cases of a more general (but less employable) equation for predicting the barriers to electron transfer (Murdoch, 1983).

Results

The $E_{1/2(subs)}$ values and the Michaelis kinetic parameters (k_{cat} and K_m) for the NADPH–P-450 reductase supported N-demethylation of the eight parasubstituted *N,N*-dimethylanilines investigated are compiled in Table I. These $E_{1/2(subs)}$ values, corrected to SCE, are within 30 mV of reported values for 4-CH_3, 4-H, 4-Cl, and 4-NO_2-substituted dimethylanilines (Seo et al., 1966). The k_{cat} values for each substrate have been determined over a sufficient range of substrate concentrations, including those near and above the determined K_m value in each instance, to elicit confidence. Less significance is attached to the K_m values, where less certainty exists because of the concentration ranges selected; further, K_m is a complex kinetic parameter that reflects the affinity of P-450 for the substrate as well as other steric, hydrophobic, and kinetic factors and may not be relevant to rates of electron transfer.

We have additionally examined an abbreviated series of para-substituted *N,N*-dimethylanilines for their rates of N-demethylation with a P-450 system in which substrate oxidation was iodosylbenzene-supported. The k_{cat} parameters for

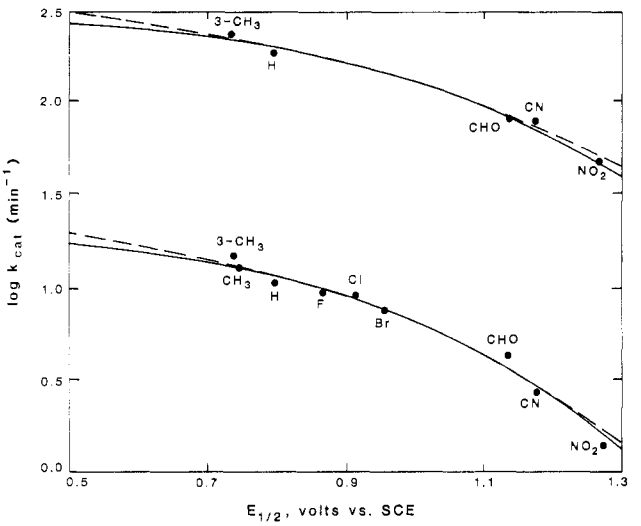

FIGURE 3: Logarithm of k_{cat} for reaction of cytochrome P-450 with *N*,*N*-dimethylanilines versus $E_{1/2(subs)}$. Except for the 3-CH_3-aniline derivative, all points represent 4-substituted *N*,*N*-dimethylanilines. The upper set of three curves represents the analysis of the iodosylbenzene-supported oxidation system, and the bottom set represents the analysis for the NADPH–P-450 reductase system. Nonlinear least-squares fits to Rehm–Weller (—) and Agmon–Levine (-·-) equations are nearly superimposable.

N-demethylation of this attenuated series of substituted dimethylanilines by the iodosylbenzene-supported system are compiled in Table I. In this system, as with other P-450-dependent systems supported by surrogate oxygen agents (see Figure 1), the high valent iron–oxo species is thought to be identical with that produced by the enzyme species through the agency of NADPH–P-450 reductase and the cofactors (Ortiz de Montellano, 1986). The rates of substrate oxidation with the iodosyl-supported system are constant for only short periods of time due to competing heme destruction, and the incubation time was adjusted accordingly. The experimental difficulty associated with conducting this protocol quantitatively, with its attendant decrease in precision, has limited our extension of this approach to all substrates.

The free energy of activation takes different expressions in the three treatments (eq 2–4). In all three equations $\Delta G^{+} = -23060(E_{1/2(subs)})$ and λ is the reorganization energy, which represents the free energy change associated with adjustments in the ligand geometries and solvent shells of the interacting electron-transfer pair required to satisfy the Frank–Codon principle [see: Eberson (1982, 1985) and McClendon (1988)]. For the curve fitting, the errors were taken as the standard deviations listed in Table I. Results of the nonlinear least-squares fitting of the three parameters A, λ, and $E_{1/2(app)}$ are shown in Table II, where $E_{1/2(app)}$ is the apparent oxidation potential of P-450. The nonlinear least-squares fitting of the relationship between log k_{cat} and $E_{1/2(sub)}$ to the Marcus, Rehm–Weller, and Agmon–Levine equations (eq 2–4) provides values for the three adjustable parameters that are nonexclusive but have low statistical error. Parameters obtained for acceptable fits for the Rehm–Weller and Agmon–Levine expressions appear in Table II. As indicated by the nearly superimposable curves in Figure 3 for both iodosylbenzene- and reductase-based systems, both the Rehm–Weller and Agmon–Levine expressions yield curves that pass near all the points. Table II shows that the pair of expressions yield virtually identical λ and $E_{1/2(app)}$ values with significant differences appearing only in the preexponential term A. The difference in $E_{1/2(app)}$ for the reductase- and iodosylbenzene-supported systems obtained from these analyses is not significant, and an average $E_{1/2(app)}$ of 1.85 V (SCE) represents both systems. In contrast, attempted fittings to the Marcus equations are unsatisfactory—to track the other two curves in Figure 3, the Marcus equation demands values of λ ranging from 70 to 100 kcal mol^{-1} and $E_{1/2(app)}$ from 4 to 5 V.

Discussion

We have determined the rates (k_{cat}) of N-demethylation of a series of substituted dimethylanilines and discovered an inverse correlation between the rate of substrate oxidation and the substrate oxidation–reduction potential ($E_{1/2(subs)}$). In an attempt to employ these data to assess the oxidation potential of the activated iron–oxo intermediate of P-450, we have analyzed the data according to the Eyring equation (eq 1), with A being a preexponential term representing all catalytic steps prior to electron transfer and ΔG^{+} representing the energy of activation for electron transfer. The kinetic scheme for the catalytic cycle of P-450 that is represented by this treatment is

$$(CH_3)_2NAr + [Fe] \rightleftharpoons \rightleftharpoons \rightleftharpoons \rightleftharpoons [Fe{=}O + (CH_3)_2NAr] \xrightarrow{k_{cat}} [Fe{-}O^{-}(CH_3)_2NAr^{\bullet+}] \rightleftharpoons \rightleftharpoons [Fe + (HOCH_2)(CH_3)NAr] \rightleftharpoons \rightleftharpoons [Fe] + HCOH + CH_3NHAr \quad (5)$$

This scheme requires assumptions about the catalytic cycle of cytochrome P-450 as it is outlined in Figure 1. The most obvious and critical assumption in this analysis is that subsequent to the formation of the activated ferryl (Fe=O)—substrate-bound enzyme intermediate, the transfer of an electron from dimethylaniline to enzyme is either the rate-determining step in the formation of the putative *N*-(hydroxymethyl)-*N*-methylaniline product or is irreversible. A body of data on the oxidation of unsaturated and heteroatom-containing substrates by P-450 is consistent with the process of oxygen transfer, including whatever electron-transfer steps are involved, being rate-determining in the oxidation of substrate (subsequent to the sequence of oxygen activation) (Ortiz de Montellano, 1986; Komives & Ortiz de Montellano, 1987; Burka et al., 1983; White & McCarthy, 1986). However, in the kinetic scheme developed to quantitatively examine the relationship between substrate $E_{1/2}$ and k_{cat} for N-demethylation, the rate for product release (k_{off}) is assumed not to contribute to the observed rate of substrate oxidation. However, the kinetic model suggests that as k_{off} increasingly contributes to rate determination for product formation, the $E_{1/2(app)}$ of the enzyme will be reduced accordingly.[2]

The approach that we have utilized in assessing the oxidation–reduction potential of P-450 follows that developed for

[2] However, current knowledge of P-450 mechanisms and analysis of the literature would argue that, in general, the rate of product dissociation is not a rate-limiting step in P-450 catalysis. For instance, the rate can be approximated by studies that estimate the rate of substrate dissociation. Rabbit $P\text{-}450_{LM2}$ has been estimated to bind benzphetamine at a rate of $\sim 5 \times 10^{6}$ min^{-1} M^{-1} (Blanck et al., 1976). An estimate of the rate as $>4 \times 10^{4}$ min^{-1} at a substrate concentration of 10^{-4} M (the K_s) gives a better estimate of $>4 \times 10^{8}$ min^{-1} M^{-1} for the substrate association rate, more consistent with the value obtained for bacterial $P\text{-}450_{cam}$ (Griffin & Peterson, 1972). Thus, a dissociation rate of $>4 \times 10^{4}$ min^{-1} (Guengerich & Coon, 1975) would be predicted (cf. Table I, Figure 3) for this tertiary amine, a reasonable model for the secondary amine products in this work. Further, rate-limiting product dissociation rates do not seem compatible with (1) the accumulation of catalytic intermediates other than the ferric product complex (Tyson et al., 1972; Guengerich et al., 1976), (2) the high kinetic deuterium isotope effects seen in many P-450-catalyzed reactions, even for intermolecular isotope effects (Ortiz de Montellano, 1986; White & Coon, 1980), and (3) the observation that iodosylbenzene can yield high oxidation rates (Table I; Ortiz de Montellano, 1986).

the estimation of the oxidation–reduction potentials of excited states by electron-transfer quenching by a series of electron donors (Ballardini et al., 1978; Bock et al., 1979). In this approach it is assumed that the preexponential term (A) for each activation protocol and the organization energy for the electron transfer from enzyme to substrate (λ) will remain approximately constant throughout the series of substituted dimethylaniline substrates, since the structural and electronic features of the enzymic oxidant and the substrates will not vary substantially upon substrate para- or meta-substitution. Several uses of this approach in chemical systems have verified the assumption that the λ values for a structurally similar series of amino-substituted aromatics are approximately constant (Ballardini et al., 1978; Bock et al., 1979; Eberson, 1982). In this analysis it is necessary that the preexponential term A be relatively invariant for each series of substrates examined by a single activation protocol.

Two protocols have been employed for the activation of P-450 to the activated iron–oxo complex—the physiological activation sequence employing NADPH–P-450 reductase/$NADPH/O_2$ and a surrogate oxygen-transfer approach that overcomes the requirement for these additional species and that utilizes iodosylbenzene as an oxygen source. The ability to investigate two distinct P-450-dependent N-demethylation reactions, with independent rates of production of the activated iron-oxo intermediate, has provided us with a method in which to decipher the contributions of the enzymatic steps preceding substrate oxidation, such as substrate introduction and production of the active oxygen intermediate. The preexponential (A), λ, and $E_{1/2(app)}$ values for each oxygenation support system are assembled in Table II, and the relationships between k_{cat} and $E_{1/2(subs)}$ for both activation systems are plotted in Figure 3.

The relationship between the rate of substrate oxidation and the substrate oxidation–reduction potential for both oxygen activation protocols has been analyzed according to three theories of electron transfer (eq 2–4), all of which can be derived from a common theoretical precursor (Murdoch, 1983). The general lack of correlation with the Marcus equation of data for *intermolecular* electron transfer has been ascribed to rate-determining reactant diffusion (Miller et al., 1984; McLendon, 1988). The Rehm–Weller and Agmon–Levine equations provide excellent correlations of the data for the parameters indicated in Table II, while the Marcus analysis does not (see Figure 3). The Marcus equation (eq 2) is derived from statistical mechanics and requires that as the electron transfer becomes extremely exothermic ($G^{+} \ll G^{\circ}$), the rate of reaction becomes slower (Marcus & Sutin, 1985; Eberson, 1982). This requirement for the Marcus "inverted region", with which many experimental observations do not agree [cf. Meyer et al. (1983), Eberson (1982), and McLendon (1988)], has led to the development of empirical approaches to correlating the rate of electron transfer with the difference in potentials of the donor–acceptor pair and an additional extrakinetic parameter. The Agmon–Levine (eq 4) and Rehm–Weller (eq 3) equations are representative of semiempirical relationships in which the predicted rate of electron transfer approaches an asymptote as the calculated exergonicity of the reaction becomes greater; the asymptote of these equations is thought to represent the diffusion-controlled rate for electron transfer in intermolecular reactions (Miller et al., 1984; McLendon, 1988). The asymptote that is approached in the case modeled here is a function of the equilibrium constants of the equilibria preceding electron transfer and depends critically on the method of oxygen activation.

We have found that the principal differences in the preexponential, reorganization energy, and $E_{1/2(app)}$ values (Table II) derived by analysis of the two activation protocols reside in the preexponential terms, which vary by approximately 17-fold for both the Rehm-Weller and Agmon–Levine analyses. The differences in the values for the preexponential term, A, derived from each equation are a function of the equations and are not relevant. However, it is significant that the difference in the three parameters between the reductase- and iodosylbenzene-supported activation protocols lies primarily in the preexponential factor, since this finding is consistent with the assumption in our analysis that oxygen activation is a preexponential contribution to substrate oxidation and that substrate oxidation is rate-determining subsequent to this process.

The analysis provides average values for λ of 23 kcal mol^{-1} and for $E_{1/2(app)}$ of 1.85 V (SCE). Support for an average reorganization energy of $\sim$23 kcal mol^{-1} for the electron transfer between para-substituted dimethylaniline substrates and a heme–oxo species in an enzyme active site can be derived from model studies. From a variety of chemical studies it is known that the λ values for *N*,*N*-dimethylaniline/*N*,*N*-dimethylaniline radical cation electron self-exchange are low ($<$15 kcal mol^{-1}) (Ballardini et al., 1978; Bock et al., 1979; Eberson, 1982). The reorganization energies for electron transfer in proteins with porphyrin prosthetic groups appear to span a considerable range. Although theoretical treatments of electron transfer in porphyrin redox proteins suggested that the reorganization energies for these processes would be low ($<\sim$8 kcal mol^{-1}; Chung et al., 1983), recent experimental data suggest that the λ value for electron transfer in natural and model bis(porphyrin) protein couples is larger [$\sim$15–20 kcal mol^{-1}; McLendon & Miller, 1985; Cheung et al., 1986; McLendon, 1988; $\sim$50 kcal mol^{-1}; Peterson-Kennedy et al., 1984; for reviews, see McLendon et al. (1985), and Peterson-Kennedy et al. (1985)]. However, all of these electron-transfer processes are "long-range", in contrast to that proceeding in the P-450 active site, and the reorganization energy is distance dependent (Marcus & Sutin, 1985). Assuming an intermediate range ($\sim$30 kcal mol^{-1}) for the self-exchange of the P-450 $(FeO)^{3+}/(FeO)^{2+}$ couple, a reorganization energy of $\sim$23 kcal mol^{-1} for the P-450-catalyzed oxidation of *N*,*N*-dimethylaniline substrates would be in excellent agreement.

The estimated $E_{1/2(app)}$ of 1.85 V (SCE) is higher than the values measured for model mangano (Bortolini & Meunier, 1983) and iron porphyrins (Lee et al., 1985; Groves & Gilbert, 1986) and the hypervalent forms of horseradish peroxidase (Hayashi & Yamazaki, 1979). These values range from 1.00 to 1.75 in the model metalloporphyrins, depending upon the solvent and ligands. The $E_{1/2(app)}$ values for oxidation–reduction of compound I and compound II of horseradish peroxidase are accessible by dye equilibration methods with the reported (pH-dependent) values being 0.75 and 0.73 V (vs SCE) at pH 7.0. Unlike some other peroxidases, horseradish peroxidase does not appear to involve tyrosinyl cations, and the measured $E_{1/2(app)}$ is believed to reflect redox changes of the heme–oxo species.

We propose that subsequent to electron transfer coulombic interactions between the charged heme–oxo radical anion–dimethylaniline radical cation pair (see eq 5 and 6) can account for the enhanced $E_{1/2(app)}$ of P-450. In our treatment, the $E_{1/2(app)}$ is the sum of an intrinsic oxidation potential, $E_{1/2(int)}$, for the $(Fe{=}O)^{3+}$ porphyrin core of the enzyme and a coulombic factor [$E_{(cf)}$], which arises from the gain or loss of electrostatic free energy upon transfer of the electron in the

transition state [see Eberson (1985)]. The free energy for the electrostatic contribution ($G_{(cf)}$) is expressed in eq 7, in which

$$E_{1/2(app)} = E_{1/2(int)} + E_{(cf)} \quad (6)$$

$$C_{(cf)} = (Z_1 - Z_2 - 1)e^2 f / r_{1,2} D \quad (7)$$

$$E_{(cf)} \text{ (expressed in V)} = +14.4/r_{1,2}D \text{ Å} \quad (8)$$

Z_1 and Z_2 are the initial charges of the enzyme active oxidant and the substrate, respectively, e is the electronic charge, f is a factor expressing the influence of ionic strength (μ) on the coulombic interaction that becomes unity near zero ionic strength, r is the center–center internuclear distance in the transition state between the nuclei of Z_1 and Z_2, and D is the static dielectric constant of the enzyme active site. Assuming the active site of P-450 has negligible ionic strength ($\mu \cong 0$) and because the substrate and iron–oxo core of the enzyme are uncharged [due to the heme-axial thiolate ligation of the $(FeO)^{3+}$ species; vide infra], $E_{(cf)}$ collapses to 14.4 Å/$r_{1,2}D$ (eq 8), by substitution in the Nernst equation. Thus, the internuclear distance for electron transfer and the dielectric constant of the P-450 active site assume crucial importance in determining the magnitude of $E_{(cf)}$.

Neither the internuclear distance ($r_{1,2}$) nor the static dielectric constant (D) of the active site are known, although reasonable estimates of these critical parameters can be advanced. For illustration, a distance of 5 Å between the charged centers (the nuclei of the negatively charged oxygen atom and the positively charged nitrogen atom) and a dielectric constant of 3 are considered. Support for a 5-Å distance approximation derives from substrate-free and -bound $P\text{-}450_{(cam)}$ in which the distance between the $(FeO)^{3+}$ oxygen and the hydrogen abstracted in camphor is postulated to be less than half of this proposed distance (Poulos et al., 1986, 1987; Poulos, 1986). Presumably, distances substantially greater than 5 Å (e.g., 20 Å) are not realistic in the active site. Controversy currently surrounds the estimation of dielectric constants in proteins [for conflicting viewpoints, see Gilson and Honig (1986, 1987), Warshel and Russell (1984), and Warshell (1987)], since the range of potential values (2.0–80 or higher) has a dramatic impact on the magnitude of electrostatic interactions in proteins, which are thought to affect both protein structure and function. To illustrate the size of the postulated coulombic interaction in P-450 electron transfer, we utilize a dielectric constant of 3. This value has been theoretically estimated to be the effective dielectric constant for a folded protein at internal distances greater than 5 Å from the protein–water interface (Gilson & Honig, (1986) and is intermediate between the 2.0 (Gilson & Honig, 1987) and 3.5 values (Sternberg et al., 1987) typically employed for proteins. At these postulated values for the distance of electron transfer and the active-site dielectric constant, $E_{(cf)}$ corresponds to 1 V, which would serve to enhance the intrinsic oxidation potential of the enzyme. A decrease in the dielectric constant to 2 increases $E_{(cf)}$ to ~1.5 V; increases in either the distance or dielectric constant serve to correspondingly reduce the magnitude of $E_{(cf)}$.

We therefore conclude that in P-450 catalytic processes initiated by electron transfer coulombic attraction of the ion pair produced by this reaction contributes significantly to the $E_{1/2(app)}$ of the enzyme. At present the contributions to the effective oxidation potential of P-450 by the intrinsic oxidation potential of the $(FeO)^{3+}$ species and the coulombic factor cannot be rigorously dissected. Nonetheless, the coulombic contribution must be significant if model studies are our guide. For example, if the 1.0–1.3-V estimates of the oxidation potential of the $(FeO)^{3+}$ core of the enzyme derived from model studies are reasonable, the magnitude of the electrostatic interaction is postulated to be approximately 0.5–0.8 V. The proposal of a coulombic interaction in P-450-mediated electron-transfer reactions is dependent upon the overall charge neutrality imparted to the $(FeO)^{3+}$ species by the cysteinyl thiolate ligand and the heme dianionic ligand. Thus, the unique chemistry of P-450, long presumed to be a function of the unusual axial thiolate ligand of this hemoprotein (Dawson & Sono, 1987; Dawson, 1988; Ortiz de Montellano, 1987), may be in part due to the overall charge neutrality of the high valent heme–oxo species afforded by this ligand. The axial thiolate ligation would also serve to localize anionic charge on the ferryl oxygen subsequent to electron transfer [see Dawson and Sono (1987) and Dawson (1988)], which would additionally serve to enhance the coulombic interaction. These properties of the P-450 $(FeO)^{3+}/(FeO)^{2+}$ couple conferred by the axial thiolate ligand have not been previously recognized and imply an additional role for this ligand in the chemistry of P-450.

The proposal of an effective oxidation potential for P-450 of ~1.85 V (SCE) and of a significant contribution to this potential from a coulombic interaction has important implications for the mechanisms of P-450-catalyzed oxidations of heteroatom-containing and unsaturated substrates and for model systems that mimic the enzyme. For example, such electrostatic interactions may not be significant in the reactions of model systems or in enzymatic processes that proceed at the protein–water interface, since the oxidant may not be neutral or the dielectric constant of the media may be sufficiently large (>20). Thus, the effective oxidation potential of these oxidants would not be enhanced by $E_{(cf)}$ and consequently not represent ideal models of the enzymatic transformations of P-450. In addition, the proposal of an $E_{1/2(app)}$ of ~1.85 V for P-450 dramatically broadens the scope of substrates that are potential substrates for enzymatic oxidation by initial electron transfer. Experimental data are consistent with a role of P-450 in the single electron transfer oxidation of substrates with a range of oxidation–reduction potentials from amines ($E_{1/2}$ = ~1.0 V) to ethers and iodides ($E_{1/2}$ = <~2.1 V) (Guengerich et al., 1984). Indeed, with the unique ligand structure surrounding the heme of P-450, nature may have crafted a potent oxidant designed to function by single electron transfer.

Registry No. P-450, 9035-51-2; monooxygenase, 9038-14-6; 3-CH_3-*N*,*N*-dimethylaniline, 121-72-2; 4-CH_3-*N*,*N*-dimethylaniline, 99-97-8; 4-H-*N*,*N*-dimethylaniline, 121-69-7; 4-F-*N*,*N*-dimethylaniline, 403-46-3; 4-Cl-*N*,*N*-dimethylaniline, 698-69-1; 4-Br-*N*,*N*-dimethylaniline, 586-77-6; 4-CHO-*N*,*N*-dimethylaniline, 100-10-7; 4-CN-*N*,*N*-dimethylaniline, 1197-19-9; 4-NO_2-*N*,*N*-dimethylaniline, 100-23-2.

References

Augusto, O., Beilan, H. S., & Ortiz de Montellano, P. R. (1982) *J. Biol. Chem. 257*, 11288–11295.

Ballardini, R., Varani, G., Indelli, M. T., Scandola, F., & Balzani, V. (1978) *J. Am. Chem. Soc. 100*, 7219–7223.

Blanck, J., Smettan, G., Jänig, G.-R., & Ruckpaul, K. (1976) *Acta Biol. Med. Ger. 35*, 1455–1463.

Bock, C. R., Connor, J. A., Gutierrez, A. R., Meyer, T. J., Whitten, G., Sullivan, B. P., & Nagle, J. K. (1979) *J. Am. Chem. Soc. 101*, 4815–4824.

Bortolini, O., & Meunier, B. (1983) *J. Chem. Soc., Chem. Commun.*, 1364–1366.

Burka, L. T., Plucinski, T. E., & Macdonald, T. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6680–6684.

Burka, L. T., Guengerich, F. P., Willard, R. J., & Macdonald, T. L. (1985) *J. Am. Chem. Soc. 107*, 2549–2551.

Cheung, E., Taylor, K., Kornblatt, J. A., English, A. M., McLendon, G., & Miller, J. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1330–1333.
Chung, A., Weiss, R., Warshel, A., & Takano, T. (1983) *J. Phys. Chem. 87*, 1683–1694.
Cochin, J., & Alexrod, J. (1959) *J. Pharm. Exp. Ther. 125*, 105–110.
Dawson, J. H. (1988) *Science 240*, 433–439.
Dawson, J. H., & Sono, M. (1987) *Chem. Rev. 87*, 1255–1276.
Doerge, D. R. (1986) *Arch. Biochem. Biophys. 244*, 678–685.
Eberson, L. (1981) *Prog. Phys. Org. Chem. 18*, 79–186.
Eberson, L. (1985) *Adv. Free Radical Biol. Med. 1*, 19–90.
Galliani, G., Rindone, B., Dagnino, G., & Salmona, M. (1984) *Eur. J. Drug. Metab. Pharmacokinet. 9*, 289–293.
Galliani, G., Nali, M., Rindone, B., Tollari, S., Rocchetti, M., & Salmona, M. (1986) *Xenobiotica 16*, 511–517.
Gilson, M. K., & Honig, B. H. (1986) *Biopolymers 25*, 2097–2119.
Gilson, M. K., & Honig, B. H. (1987) *Nature 330*, 84–86.
Gribble, G. W., & Nutaitis, G. F. (1985) *Org. Prep. Proced. Int. 17*, 317–384.
Griffin, B. W., & Peterson, J. A. (1972) *Biochemistry 11*, 4740–4746.
Groves, J. T., & Gilbert, J. A. (1986) *Inorg. Chem. 25*, 123–125.
Guengerich, F. P. (1984) *J. Med. Chem. 27*, 1101–1103.
Guengerich, F. P. (Ed.) (1987) *Mammalian Cytochromes P-450*, Vol. 1 and 2, CRC Press, Boca Raton, FL.
Guengerich, F. P., & Coon, M. J. (1975) *Pharmacologist 17*, 216.
Guengerich, F. P., & Martin, M. V. (1980) *Arch. Biochem. Biophys. 205*, 365–379.
Guengerich, F. P., & Macdonald, T. L. (1984) *Acc. Chem. Res. 17*, 9–16.
Guengerich, F. P., & Böcker, R. H. (1988) *J. Biol. Chem. 263*, 8168–8175.
Guengerich, F. P., Ballou, D. P., & Coon, M. J. (1976) *Biochem. Biophys. Res. Commun. 70*, 951–956.
Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V., & Kaminsky, L. S. (1982) *Biochemistry 21*, 6019–6030.
Guengerich, F. P., Willard, R. J., Shea, J. P., Richards, L. E., & Macdonald, T. L. (1984) *J. Am. Chem. Soc. 106*, 6446–6447.
Guengerich, F. P., Müller-Enoch, D., & Blair, I. A. (1986) *Mol. Pharmacol. 30*, 287–295.
Hanzlik, R. P., & Tullman, R. H. (1982) *J. Am. Chem. Soc. 104*, 2048–2050.
Hayashi, Y., & Yamazaki, I. (1979) *J. Biol. Chem. 254*, 9101–9106.
Kadlubar, F. F., & Hammons, G. J. (1987) in *Mammalian Cytochromes P-450* (Guengerich, R. P., Ed.) Vol. 2, pp 81–130, CRC Press, Boca Raton, FL.
Kobayashi, S., Nakano, M., Kimura, T., & Schaap, A. P. (1987) *Biochemistry 26*, 5019–5022.
Komives, E. A., & Ortiz de Montellano, P. R. (1987) *J. Biol. Chem. 262*, 9793–9802.
Lee, W. A., Calderwood, T. S., & Bruice, T. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4301–4305.
Macdonald, T. L., Zirvi, K., Burka, L. T., Peyman, P., & Guengerich, F. P. (1982) *J. Am. Chem. Soc. 104*, 2050–2052.
Marcus, R. A., & Sutin, N. (1985) *Biochim. Biophys. Acta 811*, 265–322.
McLendon, G. (1988) *Acc. Chem. Res. 21*, 160–167.
McLendon, G., & Miller, J. R. (1985) *J. Am. Chem. Soc. 107*, 7811–7816.
McLendon, G., Guarr, T., McGuire, M., Simolo, K., Stranch, S., & Taylor, K. (1985) *Coor. Chem. Rev. 64*, 113–124.
McMurry, T. J., & Groves, J. T. (1986) in *Cytochrome P-450* (Ortiz de Montellano, R. P., Ed.) pp 1–28, Plenum Press, New York.
Meyer, T. E., Przysiecki, C. T., Watkins, J. A., Bhattacharyya, A., Simondsen, R. P., Cusanovich, M. A., & Tollin, G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6740–6744.
Meyer, T. E., Cheddar, G., Bartsch, R. G., Getzoff, E. D., Cusanovich, M. A., & Tollin, G. (1986) *Biochemistry 25*, 1383–1390.
Miller, J. R., Calcaterra, L. T., & Closs, G. L. (1984) *J. Am. Chem. Soc. 106*, 3047.
Miwa, G. T., Walsh, J. S., Kedderis, G. L., & Hollenberg, P. F. (1983) *J. Biol. Chem. 258*, 14445–14449.
Murdoch, J. (1983) *J. Am. Chem. Soc. 105*, 2159–2164.
Nash, T. (1953) *Biochem. J. 55*, 416–421.
Ortiz de Montellano, P. R. (1986) in *Cytochrome P-450* (Ortiz de Montellano, R. P., Ed.) pp 217–271, Plenum Press, New York.
Ortiz de Montellano, P. R. (1987) *Acc. Chem. Res. 20*, 289–294.
Peterson-Kennedy, S. E., McGourty, J. L., & Hoffman, B. M. (1984) *J. Am. Chem. Soc. 106*, 5010–5012.
Peterson-Kennedy, S. E., McGourty, J. L., Ho, P. S., Sutoris, C. J., Liang, N., Zemal, H., Blough, N. V., Margoliash, E., & Hoffman, B. M. (1985) *Coord. Chem. Rev. 64*, 125–133.
Poulos, T. L. (1986) in *Cytochrome P-450* (Ortiz de Montellano, R. P., Ed.) pp 503–523, Plenum Press, New York.
Poulos, T. L., Finzel, B. C., & Howard, A. J. (1986) *Biochemistry 25*, 5314–5322.
Poulos, T. L., Finzel, B. C., & Howard, A. J. (1987) *J. Mol. Biol. 195*, 687–700.
Przysiecki, C. T., Cheddar, G., Meyer, T. E., Tollin, G., & Cusanovich, M. A. (1985) *Biochemistry 24*, 5647–5652.
Seo, E. T., Nelson, R. F., Fritsch, J. M., Marcoux, L. S., Leedy, D. W., & Adams, R. N. (1966) *J. Am. Chem. Soc. 88*, 3498–3503.
Shea, J. P., Nelson, S. D., & Ford, G. P. (1983) *J. Am. Chem. Soc. 105*, 5451–5454.
Sternberg, M. J. E., Hayes, F. R. F., Russell, A. J., Thomas, P. G., & Gersht, A. R. (1987) *Nature 330*, 86–88.
Tyson, C. A., Lipscomb, J. D., & Gunsalus, I. C. (1972) *J. Biol. Chem. 247*, 5777–5784.
Warshel, A. (1987) *Nature 330*, 15–16.
Warshel, A., & Russell, S. T. (1984) *Q. Rev. Biophys. 17*, 283–441.
Watanabe, Y., Oae, S., & Iyanagi, T. (1982) *Bull. Chem. Soc. Jpn. 55*, 188–195.
White, R. E., & Coon, M. J. (1980) *Annu. Rev. Biochem. 49*, 315–356.
White, R. E., & McCarthy, M.-B. (1986) *Arch. Biochem. Biophys. 246*, 19–32.
Yasukochi, Y., & Masters, B. S. S. (1976) *J. Biol. Chem. 251*, 5337–5344.