BIOORGANIC CHEMISTRY 10, 283–289 (1981)

Electronic Factors and Lipophilicity in the Horseradish Peroxidase-Catalyzed Oxidation of *N*,*N*-Dialkylanilines with Hydrogen Peroxide and Oxygen

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Received July 22, 1980

Initial rates of *N*-dealkylation of 15 *N*,*N*-dialkylanilines with hydrogen peroxide and oxygen in the presence of the enzyme horseradish peroxidase are interpreted mainly in terms of electron availability on nitrogen. In these cases a mechanism similar to that postulated in the chemical oxidation of these substrates is suggested, and involves the formation of a cation radical. Lipophilicity acts as a limiting factor in the reaction, and highly hydrophilic and hydrophobic substrates deviate from the reactivity suggested by electronic factors toward higher and lower reactivity, respectively.

Tertiary amines have been subjected to reaction with a variety of oxidants. The reactions of cyclic amines with ClO_2 (*1*), of tertiary amines with potassium ferricyanide (*2*), and of aromatic amines with lead tetraacetate (*3*) have been studied in detail. A mechanism involving two different electron abstraction steps has been suggested:

$$R_2N-CH_2R' \xrightarrow{-e^-} R_2\overset{+\cdot}{N}-CH_2R' \qquad [1]$$

$$R_2\overset{+\cdot}{N}-CH_2R' \xrightarrow{-H^+} R_2N-\dot{C}HR' \qquad [2]$$

$$R_2N-\dot{C}HR' \xrightarrow{-e^-} R_2N-\overset{+}{C}HR' \qquad [3]$$

$$R_2N-\overset{+}{C}HR' \xrightarrow{H_2O} R_2N-H + R'CHO + H^+ \qquad [4]$$

The first electron transfer is the rate-determining step. The results of kinetic investigations of these oxidations (*1–3*) fit this mechanistic scheme, assuming that the cation radical and the α-alkylarylamino carbon radical are in a steady state. The comparison between the chemical oxidation and the enzyme-catalyzed oxidation of these substrates with oxygen is a useful tool for understanding the mechanism of the microsomal oxidation of organic nitrogen compounds.

The enzymic system we investigated was the system horseradish peroxidase–hydrogen peroxide–oxygen. This system has been indicated to work also as an oxidase (*4*), and is often regarded as a simple model for more complex biological oxidizing systems. Horseradish peroxidase (HRP) catalyzes the oxidation of a

0045-2068/81/030283-07$02.00/0

wide variety of substrates according the following scheme (*5*, *6*):

$$HRP + H_2O_2 \rightleftharpoons HRP\text{–}H_2O_2 \rightarrow E_1 \quad [5]$$

$$E_1 + AH_2 \rightleftharpoons E_1\text{–}AH_2 \rightarrow E_2 + AH^{\cdot} \quad [6]$$

$$E_2 + AH_2 \rightleftharpoons E_2\text{–}AH_2 \rightarrow HRP + AH^{\cdot} \quad [7]$$

$$2\,AH \rightarrow A_2H_2 \text{ or } A + AH_2 \quad [8]$$

Here HRP is the ferric enzyme, and E_1 and E_2 are oxidized forms of the enzyme at, respectively, two and one higher level of oxidation.

In previous investigations (*7*) we showed that HRP catalyzes the oxidation of aromatic tertiary amines with hydrogen peroxide, yielding a secondary amine and an aldehyde according to the equation

$$Ar\text{-}NRCH_2R' + H_2O_2 \rightarrow Ar\text{-}NHR + R'CHO + H_2O. \quad [9]$$

Additional work showed that the overall stoichiometry of the conversion of some *N*,*N*-dimethylanilines to the corresponding *N*-methyl derivatives is accounted for by total conversion of hydrogen peroxide *plus* partial consumption of the oxygen dissolved in the reaction medium. The nearly equal amounts (as equivalents) of oxygen and hydrogen peroxide suggested that only reaction [1] is step catalyzed by peroxidase, by action of either compound E_1 or E_2, while step 3 is performed by molecular oxygen via electron transfer from the radical species released from the enzyme into the solution. Satisfactory support for this mechanism is provided by the evidence of the superoxide anion in the system (*8*).

Here we present the results of a kinetic investigation performed for a series of 15 *N*,*N*-dimethylanilines and *N*,*N*-diethylanilines (DMA and DEA). When the concentration of substrate, hydrogen peroxide, and enzyme were kept sufficiently low, in most cases common features of peroxidase oxidations such as coupling of radical intermediates, polycondensation, and colored products could be avoided. The rate of dealkylation could be followed directly by uv spectroscopy.

Preliminary experiments showed that the dependence of the rate of demethylation of some *N*,*N*-dimethylanilines on hydrogen peroxide concentration was defined by a typical Michaelis–Menten kinetic pattern. Slight inactivation of the enzyme was noted at concentrations of hydrogen peroxide higher than necessary to reach the saturation plateau. Oxygen concentration could be lowered as low as 10^{-4} *M* without any appreciable effect on the rate of demethylation. Kinetic experiments were therefore performed at normal oxygen concentration (2.4×10^{-4} *M*) without any adjustment. Substrate concentrations ranged from 5×10^{-5} to 10^{-3} *M*. Within this range at fixed enzyme and H_2O_2 concentration the dependence of initial rates of dealkylation upon substrate concentration was fairly linear. Only in a few cases was slight deviation from linearity observed at the upper limit of the range, in line with a Michaelis–Menten pattern where very high K_m's hold. The apparent first-order constants (k_{app}) calculated from the range where linearity holds are summarized for 15 compounds in Table 1. Here also Hammett's σ and Hansch's π lipophilicity parameters (*9*) are reported.

Some other substances were tested, but appreciable amounts of polymeric

TABLE 1

APPARENT FIRST-ORDER CONSTANTS FOR THE OXIDATIVE DEALKYLATION OF DIALKYLARYLAMINES $X-C_6H_4-NR_2$ WITH H_2O_2, O_2, AND HRP AT 25°C

R	X	λ(nm)	$k_{app} \times 10^3/sec^{-1}$	σ	π
Methyl	4-Fluoro	290	0.41	0.06	0.14
Methyl	3-Chloro	270	0.24	0.37	0.71
Methyl	4-Chloro	275	0.37	0.23	0.71
Methyl	3-Methyl	285	1.30	−0.07	0.56
Methyl	4-Isopropyl	290	0.04	−0.15	1.53
Methyl	4-Acetylamino	350	6.46	0.00	−0.97
Methyl	4-Cyano	210	0.02	0.66	−0.57
Methyl	3-Nitro	205	0.02	0.71	−0.28
Ethyl	4-Fluoro	220	1.32	0.06	0.14
Ethyl	3-Chloro	250	0.69	0.37	0.71
Ethyl	4-Chloro	230	1.07	0.23	0.71
Ethyl	3-Methyl	283	2.92	−0.07	0.56
Ethyl	4-Isopropyl	280	0.02	−0.15	1.53
Ethyl	3-Methoxy	280	1.89	0.12	−0.02
Ethyl	4-Acetylamino	245	13.55	0.00	−0.97

material were formed even under mild conditions, making kinetic measurements impossible. This is the case with *N,N*-dimethyl- and *N,N*-diethylaniline (where the *para* position is probably activated toward electrophilic attack of the radical and ionic species produced during the reaction) and the 3- and 4-methoxy derivatives.

Inspection of the experimental data allows some conclusions to be drawn about the reaction mechanism. The electronic effect in this reaction can be evaluated by observing the dependence of the logarithm of k_{app} on Hammett's σ. This is shown in Fig. 1. As a first observation, in almost all cases DEAs react faster than DMAs. This finding is in line with kinetic results obtained in the oxidations with lead tetraacetate (*3*) and suggests that an electronic factor related to a stabilization of a positive charge on nitrogen in the transition state is operating.

If one observes the region ranging from $\sigma = -0.07$ to $\sigma = 0.77$, a linear correlation for DMAs and DEAs can be seen, suggesting that in this region the electronic effect plays an important role. The analysis of the correlation holding in this range shows that the 4-acetylamino group leads to deviation from a linear correlation in both series. If it is excluded from calculations, a $\rho = -2.83$ for DMAs and -1.33 for DEAs is obtained. These values, and in particular their negative sign, suggest a transition state structure bearing a high degree of positive charge on nitrogen. Thus the mechanism via the cation radical Eqs. [1]–[4] can be proposed to be operative in the oxidation of these substrates with $HRP-H_2O_2-O_2$. In fact a ρ value of -1.83 has been reported in the photoreduction of aromatic ketones with aromatic amines (*10*), and ρ values of -0.989 and -0.924 were obtained in ferricyanide (*2*) and ClO_2 (*1*) oxidation of tertiary amines. We obtained ρ value of -2.4 in the oxidation of six *N,N*-dimethylanilines with lead

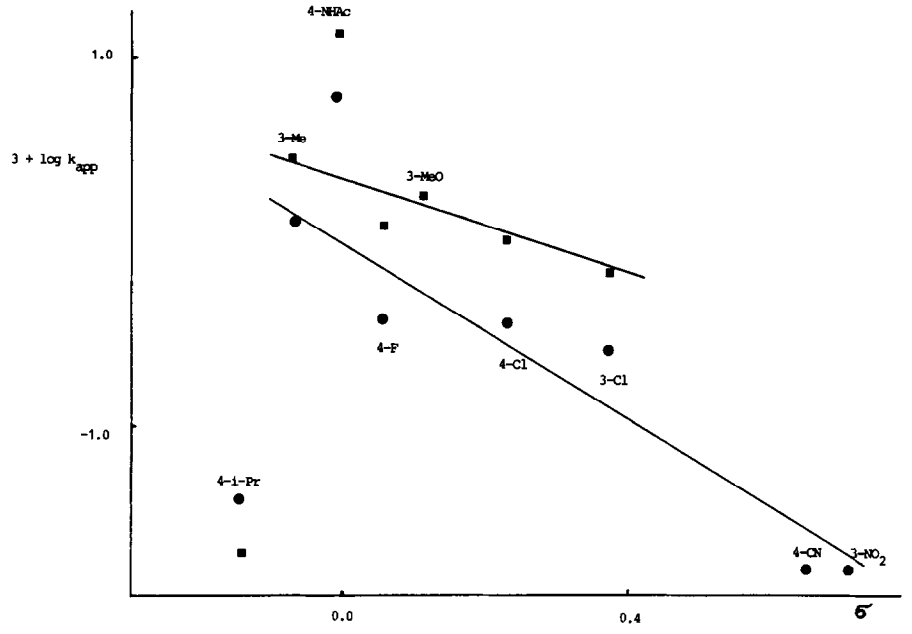

FIG. 1. Plot of the logarithm of k_{app} vs σ. ●, *N,N*-dimethyl; ■, *N,N*-diethyl.

tetraacetate and −3.5 in the oxidation of four *N,N*-dimethylanilines with thallium triacetate and oxygen (*3, 11*). All these reactions were suggested to occur through the intermediate formation of a cation radical with the assumption of a steady state.

The deviation of some substrates from the behavior predicted by their Hammett values suggests that additional factors have to be taken into account for understanding the overall reactivity. The lipophilicity factor is often important in defining the reactivity of organic compounds in enzyme-mediated reactions. This factor is suggested to contribute to the oxidative dealkylation of *N,N*-dimethylanilines with microsomes from the hepatic cell of rats pretreated with the *P*-450 cytochrome inducer phenobarbital. Also electronic and steric effects take part to define the reactivity profile in this case (*12*).

Lipophilicity (π) can be expressed as the logarithm of the partition coefficient between water and *n*-octanol of a substituted benzene divided by the same coefficient for benzene. If log k_{app} is plotted against π (Fig. 2) it can be noted that the linear correlation observed with electronic factors is confined in the narrow lipophilicity range from −0.02 to 0.71. Outside this region the correlation does not hold. In fact the highly hydrophilic 4-acetylamino group results in faster reaction than as expected, and the strongly lipophilic 4-isopropyl group gives rise to a very low reactivity. This allows their exclusion from the linear correlation with σ to be made. Such behavior could be attributed to the presence, in these cases, of a rate-determining step different from that occurring with the other substrates. As the rate-determining step has to be found among the reactions depicted by Eqs. [6] and [7], it could be the case that important variations occur in the ratio between

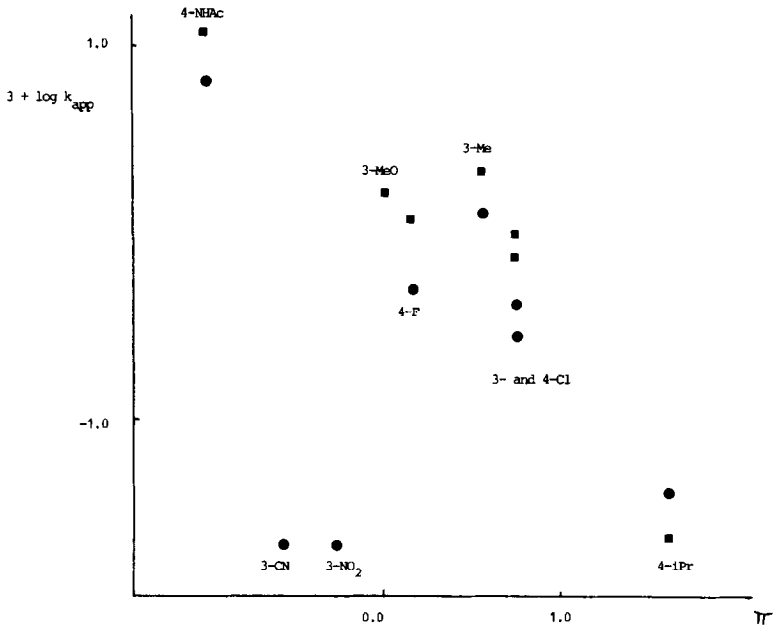

FIG. 2. Plot of the logarithm of k_{app} vs π. ●, *N*,*N*-dimethyl; ■, *N*,*N*-diethyl.

the rate of binding of the substrate to the enzyme and the rate of the release of oxidized products from the enzyme. Strong changes in lipophilicity could give rise to this situation.

The 4-cyano and 3-nitro groups are at the limits of sensitivity of the analytical method. In these cases low lipophilicity is coupled with low electron availability. We propose that these substrates, although reactive from the standpoint of lipophilicity, have too low an electron availability for the reaction to occur. A interesting feature of Fig. 1 is the inversion of reactivity of the pair *N*,*N*-dimethyl and *N*,*N*-diethylaniline both bearing the 4-isopropyl group. This behavior is in accord with the hypothesis that above a certain limit, lipophilicity is by far the most important factor affecting reactivity. In the case of the isopropyl derivative, substitution of methyl with ethyl groups results in an overall effect where exceeding lipophilicity operates; whereas in the other cases such a substitution does not exceed this limit, and the main effect is making the nitrogen atom more reactive for its greater electron availability.

Some conclusions can be drawn from these considerations. Although a reaction catalyzed by an enzyme can seldom be interpreted with the same concepts and approximations used with much less complex chemical reactions, this study shows that a linear free energy relationship (LFER) can be observed if enough simple examples are chosen, and if one investigates a region where other factors affecting the reactivity are confined to side effects or can be assumed as constant. When this requirement is fulfilled, the HRP-catalyzed oxidation of aromatic tertiary amines allows a correlation to be observed which is similar to those obtained in the chemical oxidation of these substrates.

Attempts to combine lipophilicity, electronic, and steric effects to obtain the experimental reactivity profile by multiple correlation (*9*) were also made, but no significant improvement in the correlation was obtained. This contributes to the hypothesis that "deviating" cases can be explained by the presence of a different rate-determining step.

The results obtained by Dunford and co-workers in the stopped flow kinetic study on the substituent effect on the oxidation of phenols and anilines with HRP compound I showed that also for these classes of compounds a mechanism is involved where a slow electron transfer from substrate to compound I is followed by the fast loss of a proton (*13*). Quite different results were obtained in the oxidation of anilines to nitroso compounds by chloroperoxidase and hydrogen peroxide (*14*), where a two-electron transfer process is involved and the reactivity profile seems to be determined mainly be steric factors.

EXPERIMENTAL

The reactions were performed in a buffer solution made up with $NaH_2PO_4 \times H_2O$ (5.4 g liter^{-1}) and $Na_2HPO_4 \times 12\ H_2O$ (2.7 g liter^{-1}) at pH 7.6. Horseradish peroxidase was purchased from Boehringer and was Grade II. It was used without further purification. Hydrogen peroxide was a Baker-analyzed reagent, and was tested iodometrically (*15*). *N*,*N*-Dialkylanilines were prepared from commercial anilines and dimethyl or diethylsulfate, and purified by silica gel chromatography. *N*-Methyl and N-ethyl derivatives were prepared by reduction of formyl and acetyl derivatives with lithium aluminum hydride, and purified by silica gel chromatography. Absorbances were measured with a HitachiPerkin–Elmer 124 thermostatted spectrophotometer at 25°C. For a preliminary study of the dependence of rates of oxidation on oxygen concentration, this was measured with a Clark electrode. Working curves were drawn for each couple *N*,*N*-dialkyl *N*-monoalkyl at the wavelength where differences of absorbances were at a maximum. Wavelengths are reported in Table 1.

General procedure for kinetic experiments. Freshly prepared 1% (w/v) solutions of HRP, previously tested for their activity, were added to a solution of amine at a final enzyme concentration of 45 U liter^{-1} (25°C, guaiacol and hydrogen peroxide as substrates). Reactions were started by adding 10 μl of an opportunely diluted solution of hydrogen peroxide to 3 ml of a solution of substrate and enzyme, at a final concentration of hydrogen peroxide of 4.4×10^{-5} *M*. Initial rates were correlated with substrate concentrations by the least-squares method. The slope of this correlation gives the values defined as k_{app}.

ACKNOWLEDGMENTS

This work was supported by a grant from CNR. We thank Miss M. Beretta and Mrs. R. Castellano Visaggi for their collaboration in the experimental work.

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