Morita, F. (1967) *J. Biol. Chem. 242*, 4501–4506.
Nauss, K., Kitagawa, S., & Gergely, J. (1969) *J. Biol. Chem. 244*, 755–765.
Nehei, T., Mendelson, R. A., & Botts, J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 274–277.
Perkins, W. J., Wells, J. A., & Yount, R. G. (1984) *Biochemistry 23*, 3994–4002.
Prince, H. P., Trayer, H. R., Henry, G. D., Trayer, I. P., Dalgarno, D. C., Levine, B. A., Cary, P. D., & Turner, C. (1981) *Eur. J. Biochem. 121*, 213–219.
Provencher, S. W. (1976a) *Biophys. J. 16*, 27–41.
Provencher, S. W. (1976b) *J. Chem. Phys. 64*, 2772–2777.
Reedy, M. K., Holmes, K. C., & Tregear, R. T. (1965) *Nature (London) 207*, 1276–1280.
Seidel, J., & Gergely, (1973) *Arch. Biochem. Biophys. 158*, 853–863.
Shriver, J. W. (1984) *Trends Biochem. Sci. 9*, 322–328.
Shriver, J. W., & Sykes, B. D. (1981a) *Biochemistry 20*, 6357–6362.
Shriver, J. W., & Sykes, B. D. (1981b) *Biochemistry 20*, 2004–2012.
Snedecor, G. W., & Cochran, W. G. (1967) in *Statistical Methods*, p 116, Iowa State University Press, Ames, IA.
Svensson, E. C., & Thomas, D. D. (1988) *Biophys. J. 50*, 999–1102.
Taylor, E. W. (1979) *CRC Crit. Rev. Biochem. 6*, 103–164.
Thomas, D. D., & Cooke, R. (1980) *Biophys. J. 32*, 891–906.
Tokunaga, M., Sutoh, K., Toyoshima, C., & Wakabayashi, T. (1987) *Nature (London) 329*, 635–638.
Trentham, D. R., Bardsley, R. G., Eccleston, J. F., & Weeds, A. G. (1972) *Biochem. J. 126*, 635–644.
Walker, M., & Trinick, J. (1988) *J. Muscle Res. Cell Motil. 9*, 359–366.
Webb, M. R., Hibberd, M. G., Goldman, Y. E., & Trentham, D. R. (1986) *J. Biol. Chem. 261*, 15557–15564.
Weeds, A. G., & Taylor, R. S. (1976) *Nature (London) 257*, 54–56.
Wells, J. A., & Yount, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966–4970.
Wells, C., & Bagshaw, C. R. (1984) *J. Muscle Res. Cell Motil. 5*, 97–112.
Werber, M. M., Szent-Gyorgi, A. G., & Fasman, G. (1972) *Biochemistry 11*, 2872–2883.
Yanagida, T. (1981) *J. Mol. Biol. 146*, 539–560.

Kinetic and Molecular Orbital Studies on the Rate of Oxidation of Monosubstituted Phenols and Anilines by Horseradish Peroxidase Compound II†

Junji Sakurada,‡ Reiko Sekiguchi, Koichi Sato, and Toichiro Hosoya*
Faculty of Pharmaceutical Sciences, Chiba University, Chiba 260, Japan
Received June 28, 1989; Revised Manuscript Received December 12, 1989

ABSTRACT: The second-order rate constant (k_4) for the oxidation of a series of aromatic donor molecules (monosubstituted phenols and anilines) by horseradish peroxidase (HRP) compound II was examined with a stopped-flow apparatus. The electronic states of these substrates were calculated by an ab initio molecular orbital method. It was found that in both phenols and anilines log k_4 values correlate well with the highest occupied molecular orbital (HOMO) energy level and the lowest unoccupied molecular orbital (LUMO) energy level, but not with the net charge or frontier electron density on atoms of these molecules. The HOMO and LUMO energy levels of phenols and anilines further showed linear relationships with Hammett's σ values with negative slopes. Similar results were obtained in the oxidation of substrates by HRP compound I, except that the rate of reaction was much higher than in the case of HRP compound II. In addition, the rates of oxidation of phenols by compound I or II were found to be about 1000 times higher than those of anilines with similar HOMO energy levels. On the basis of these results, the mechanism of electron transfer from the substrate to the heme iron of HRP compound II is discussed.

It is well established that horseradish peroxidase (HRP)[1] catalyzed reactions proceed in the consecutive steps (Chance, 1951; George, 1952)

$$E + H_2O_2 \xrightarrow{k_1} ES_I \quad (1)$$

$$ES_I + AH_2 \xrightarrow{k_7} ES_{II} + AH^\bullet \quad (2)$$

$$ES_{II} + AH_2 \xrightarrow{k_4} E + AH^\bullet \quad (3)$$

$$AH^\bullet + AH^\bullet \rightarrow A_2H_2 \text{ (or } A + AH_2) \quad (4)$$

where E, ES_I, and ES_{II} are the native enzyme, compound I, and compound II, respectively, and AH_2 and $AH^\bullet$ are the hydrogen donor (the second substrate) and its free radical, respectively. It was reported that the reactions between compound I and AH_2 and between compound II and AH_2 are of the second order and that the rate constant of reaction 2, k_7, is usually much higher than that of reaction 3, k_4 (Chance, 1951; Dunford & Stillman, 1976). However, little is known about the mechanism of electron transfer from the substrate to compound I and compound II.

It was shown recently that cytochrome *c* peroxidase forms a 1:1 complex with cytochrome *c* and that the hemes of the two proteins may be almost parallel with an edge separation of 16.5 Å, suggesting that the electron transfer takes place through an intricate bridge of interaction, ionic interactions, and hydrogen bonds connecting the two hemes (Poulos &

† This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.
* Address correspondence to this author at the Laboratory of Biophysical Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, Yayoi-Cho 1-33, Chiba, Chiba 260, Japan.
‡ Present address: Department of Bacteriology, Jikei University School of Medicine, Minato-ku, Tokyo 105, Japan.

[1] Abbreviations: HOMO, highest occupied molecular orbital; HRP, horseradish peroxidase; LUMO, lowest unoccupied molecular orbital.

0006-2960/90/0429-4093$02.50/0 © 1990 American Chemical Society

Kraut, 1980). On the other hand, we presented evidence indicating that the aromatic donor molecule binds to HRP in the vicinity of the heme peripheral 8-methyl group and almost perpendicular to the heme plane (Sakurada et al., 1986). This leads to the supposition that electron transfer from the substrate to the heme iron of compound II may occur at the binding site, but it is not clear how the electron transfer takes place. Since many phenols and aromatic amines are oxidizable by compound II, studies of the effect of the substituents on the rate of reaction may shed some light on this problem.

Bordeleau and Bartha (1972) suggested, on the basis of overall reaction rates of oxidation of various aniline derivatives catalyzed by a peroxidase contained in the soil fungus *Geotricum candidum*, that susceptibility to enzymic transformation depends on the electron density on the nitrogen atoms of amino groups of these substrates. Our results obtained by molecular orbital calculations, however, did not support the view, demonstrating that the rate of reaction is dependent on HOMO energy levels (Hosoya et al., 1983). On the other hand, Dunford and Adeniran (1986) reported that logarithms of k_4 values for the oxidation reaction of many phenol and aniline derivatives by HRP compound II correlate well with Hammett's σ values. In the case of the enzyme in the fungus, the rate of oxidation obtained was not for the elementary reaction 3, but rather for the overall reaction (Bordeleau & Bartha, 1972). Furthermore, the molecular orbital calculation for these substrates was performed by a semiempirical molecular orbital method (Hosoya et al., 1983). In the present paper, therefore, we have attempted to examine whether there is any correlation between the rate constant for reaction 3, k_4, of many phenol and aniline derivatives and quantum chemical indices of these substrates calculated by ab initio molecular orbital method, hoping to obtain some clue to the mechanism of the electron transfer in the peroxidase reaction.

Experimental Procedures

Materials. HRP (isozyme B + C) was purified by the method of Aibara et al. (1982), starting from crude materials with 1.0–2.0 of RZ (ratio of A_{403}/A_{280}) obtained from Wako Pure Chemical Co. The RZ of the final preparation was over 3.0. The enzyme concentration was determined spectrophotometrically at 403 nm by using a molar absorptivity of 1.02×10^5 M^{-1} cm^{-1} (Aibara et al., 1982). A fresh HRP solution was mixed with 1 equiv of H_2O_2, and the solution stood overnight at 4 °C to recover the native enzyme. Compound II was prepared from the HRP solution by adding 1 equiv of H_2O_2 and 1 equiv of $K_4Fe(CN)_6$. The concentration of the resultant solution of compound II was between 2 and 4 μM. Solutions of most substrates were prepared by dissolving a weighed amount of the compound in an appropriate volume of purified water each day to make fresh stock solutions of 1–10 mM concentration. The solutions were stored in brown bottles to prevent photochemical reactions. The substrate solution for each run was then made by diluting portions of the stock solution to give concentrations of 20–100 μM, with a sodium phosphate buffer of pH 7.0. Usually more than 10-fold excess of substrate over the enzyme was used so that the reaction occurred by a pseudo-first-order process.

Kinetic Experiments. The kinetic experiments were performed in a Hitachi double-wavelength double-beam spectrophotometer, Model 557, equipped with a stopped-flow apparatus (Hitachi 557-0814) and a personal computer (NEC PC-9801F2). The wavelengths used were λ_1 = 418 nm and λ_2 = 403 nm. One of the drive syringes of the stopped-flow apparatus was filled with the substrate, and the other syringes were filled with compound II freshly prepared as described above. The reaction was then followed by monitoring the disappearance of compound II as described (Dunford & Adeniran, 1986). Usually 10 experiments were conducted for each substrate concentration and the data stored directly in an on-line computer memory. For the experiments on the substrate with a low k_4 value, 3 mL of a freshly prepared solution of compound II in the sodium phosphate buffer was measured into a cuvette and placed in the observation chamber. The reference was filled with the buffer. A calculated amount of the stock solution of the substrate was measured and mixed rapidly with the enzyme, and the instrument was started as soon as possible. The relative absorbance at regular time intervals was then measured manually from the trace obtained from the spectrometer. The observed pseudo-first-order rate constant, k_{obs}, for each experiment, either on the stopped-flow apparatus or on the recorder of the spectrophotometer, was calculated through the use of a Guggenheim plot in the manner described by Hiromi (1979).

Quantum Chemical Calculations. Geometry and electronic states of phenol and aniline derivatives were calculated by ab initio molecular orbital method (GAUSSIAN 82 with a STO-3G basis set) (Binkley et al., 1982) using a HITAC M-680 in the Computer Center, Institute of Molecular Science, Okazaki, Japan.

Table I: Values of k_4 for Phenol and Aniline Derivatives and Hammett's σ Values for the Substituents

substituents	k_4 (M^{-1} s^{-1}) phenol and its derivatives	k_4 (M^{-1} s^{-1}) aniline and its derivatives	Hammett's σ value[a]
H	$(3.15 \pm 0.03) \times 10^5$	$(8.59 \pm 0.20) \times 10^4$	
p-CH_3	$(1.06 \pm 0.02) \times 10^6$	$(6.10 \pm 0.19) \times 10^5$	−0.17
p-Cl	$(1.10 \pm 0.02) \times 10^6$	$(1.16 \pm 0.05) \times 10^3$	0.23
p-OCH_3	$(5.95 \pm 0.11) \times 10^6$	$(6.67 \pm 0.09) \times 10^6$	−0.27
p-CHO	$(5.27 \pm 0.11) \times 10^3$	ND[b]	0.43
p-CN	$(9.25 \pm 0.20) \times 10^3$	ND[b]	0.66
m-CH_3	$(3.96 \pm 0.05) \times 10^5$	$(1.79 \pm 0.03) \times 10^5$	−0.07
m-Cl	$(6.80 \pm 0.10) \times 10^4$	$(1.52 \pm 0.03) \times 10^3$	0.37
m-OCH_3	$(1.01 \pm 0.02) \times 10^5$	$(6.26 \pm 0.13) \times 10^4$	0.12
m-CHO	$(4.56 \pm 0.11) \times 10^3$	ND[b]	0.36
m-CN	$(1.47 \pm 0.06) \times 10^2$	ND[b]	0.56
m-OH	$(3.52 \pm 0.03) \times 10^5$	ND[b]	0.12
o-CH_3	$(8.13 \pm 0.12) \times 10^4$	$(1.38 \pm 0.02) \times 10^5$	
o-Cl	$(4.42 \pm 0.05) \times 10^5$	$(1.01 \pm 0.03) \times 10^3$	
o-OCH_3	$(2.79 \pm 0.37) \times 10^5$	$(2.90 \pm 0.08) \times 10^6$	
o-CHO	$(2.73 \pm 0.04) \times 10^3$	ND[b]	
o-OH	$(4.36 \pm 0.05) \times 10^5$	ND[b]	
o-CN	$(8.71 \pm 0.17) \times 10^2$	ND[b]	
o-SH	ND[b]	$(1.36 \pm 0.01) \times 10^6$	

[a] Taken from Exner (1972). [b] Not determined.

Results

The k_4 values of many monosubstituted phenols and anilines were determined at pH 7.0 at 22 °C by the method described above and are given in Table I. The values of some substrates that were already measured by Dunford and Adeniran (1986) are generally in agreement with those obtained here.

The electronic states of these substrates were calculated by an ab initio molecular orbital method. In this calculation, only unprotonated forms were employed, because it was already confirmed that these are reacting species (Dunford & Adeniran, 1986). In the case of aniline derivatives, as shown in Figure 1, plots of HOMO energy against log k_4 show a linear relationship, the correlation coefficient being very high (r = 0.989). When these substrates were divided into para-, meta-, and ortho-substituent homologous series, the correlation coefficients were slightly increased in para- and meta-substituted compounds. In the case of phenols, the correlation coefficient between HOMO energy and log k_4 for phenols was rather low as compared with that for anilines, being 0.641.

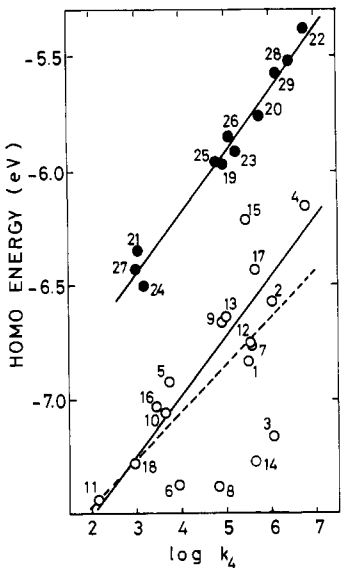


FIGURE 1: Relationship between the HOMO energy and the logarithms of k_4 of monosubstituted phenols and anilines. (○) Phenols: (1) H; (2) *p*-CH_3; (3) *p*-Cl; (4) *p*-OCH_3; (5) *p*-CHO; (6) *p*-CN; (7) *m*-CH_3; (8) *m*-Cl; (9) *m*-OCH_3; (10) *m*-CHO; (11) *m*-CN; (12) *m*-OH; (13) *o*-CH_3; (14) *o*-Cl; (15) *o*-OCH_3; (16) *o*-CHO; (17) *o*-OH; (18) *o*-CN. (●) Anilines: (19) H; (20) *p*-CH_3; (21) *p*-Cl; (22) *p*-OCH_3; (23) *m*-CH_3; (24) *m*-Cl; (25) *m*-OCH_3; (26) *o*-CH_3; (27) *o*-Cl; (28) *o*-OCH_3; (29) *o*-SH. The calculations including and excluding (3), (8), and (14) are shown by the dotted line and the full line, respectively.

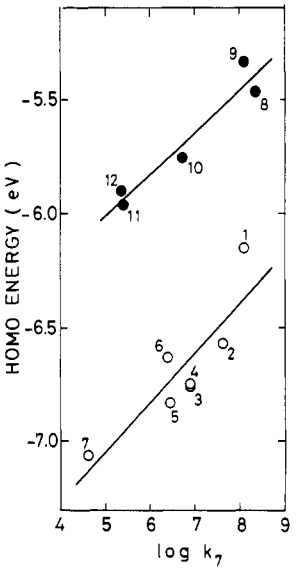
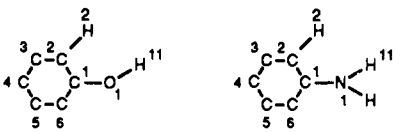


FIGURE 2: Relationship between the HOMO energy and the logarithms of k_7 of substituted phenols and anilines. The data of k_7 were taken from Job and Dunford (1976): (○) Phenols: (1) *p*-OCH_3; (2) *p*-CH_3; (3) *m*-CH_3; (4) *m*-OH; (5) H; (6) *m*-OCH_3; (7) *m*-CHO. (●) Anilines: (8) *p*-OH; (9) *p*-OC_2H_5; (10) *p*-CH_3; (12) H; (13) *m*-OC_2H_5. $r = -0.875$ ($n = 7$) for phenols; $r = -0.963$ ($n = 5$) for anilines.

Table II: Correlation Coefficients between log k_4 and Quantum Chemical Indices Obtained by the ab Initio Molecular Orbital Method



line	log k_4 versus	correlation coefficient: phenol and its derivatives (18)[a]	correlation coefficient: aniline and its derivatives (11)[a]
		Energy Level of	
A	HOMO	0.641, *0.873*[b]	0.989
B	LUMO	0.832, *0.889*[b]	0.828
		Net Charge on	
C	O1 or N1	0.005	0.349
D	C1	0.432	0.902
E	C2	0.083	0.049
F	C4	−0.310	−0.232
G	C6	−0.143	−0.066
H	H2	0.445	0.783
I	H11	0.447	0.449
		Frontier Electron Density [f_r(E)]	
J	O1 or N1	−0.024	−0.611
K	C1	−0.055	0.628
L	C2	−0.010	−0.367
M	C4	−0.321	−0.476
N	C6	−0.119	−0.023

[a] Number in parentheses denotes the number of substrates used. [b] Number in italics denotes the values obtained excluding chloro compounds.

However, if chloro derivatives, which are known to often show irregularity (Job & Dunford, 1976), were excluded, the correlation coefficient for phenols was increased to 0.873 (Table II).

From the calculations it was also found that the line for phenols was almost parallel to that for anilines, the latter being about 0.81 eV higher than the former (Figure 1), and that LUMO energy level correlates well with log k_4 in both phenol and aniline derivatives.

The quantum chemical calculation also disclosed that the net charges on oxygen atoms of phenols and on nitrogen atoms of anilines were scarcely changed by introduction of these substituents. In addition, as shown in Table II, no significant correlation was found except in the cases of C1 and H2 in anilines. Even the high correlation coefficients obtained in the three cases are considered to have been brought about eventually, since the net charges at these atoms scarcely varied among the series of compounds.

It was noteworthy that electrons of carbon atoms and oxygen or nitrogen atoms are not present in 2s, 2px, and 2py orbitals but exclusively in 2pz (2pπ) orbitals. The absolute values of coefficients of 2pz orbitals at the heteroatoms were in the range 0.2979–0.4696 in phenols and 0.4628–0.5726 in anilines, being higher than those at most carbon atoms. From these coefficients, frontier electron densities of HOMO eigenfunctions, f_r(E), were calculated according to Fukui's (1970) method and used to examine the correlation with log k_4. However, the data did not show any correlation between them (Table II, lines J–N). We have also calculated frontier electron densities f_r(R) which are related to radical reactions, but no correlation was found between log k_4 and f_r(R) (data not shown).

Previously, Job and Dunford reported the values for the rate constant (k_7) for the oxidation of various phenols and aromatic amines by compound I. Since electronic states of some of substrates were calculated as described above, other substrates except chloro derivatives and disubstituted substrates in the list (Table II) of Job and Dunford (1976) were subjected to ab initio molecular orbital calculations. The results shown in Figure 2 indicate that log k_7 values also correlate well with HOMO energy levels of substituted phenols and anilines and that the slopes of two lines are similar to each other. As in the case of log k_4 mentioned above, there is little correlation between log k_7 and net charges and frontier electron densities.

Finally, we have examined whether k_4 values for the aniline and phenol derivatives fit the Hammett equation

$$\log (k_X/k_H) = \rho\sigma \quad (5)$$

where k_X and k_H are the second-order rate constant for substituted and unsubstituted phenol and aniline and ρ and σ are the rate constant and substituent constant of the Hammett equation, respectively. The plots of log k_X/k_H against σ values yielded straight lines, from which the correlation coefficient and ρ values for phenols were found to be −0.85 and −3.78 and those for anilines, −0.96 and −5.75, respectively.

Discussion

Previously, the second-order rate constant (k_4) for the oxidation of various aromatic donor molecules by HRP compound II was obtained with a stopped-flow apparatus by several groups of investigators (Chance, 1951; Marklund et al., 1972; Yamazaki & Yokota, 1973; Dunford & Stillman, 1976; Kato et al., 1984). However, the results are not necessarily in agreement with each other, due to differences in the reaction conditions and apparatus used, and involve data of insufficient accuracy. Therefore, we have attempted to obtain the rate constants for many substrates under specific conditions.

In previous papers (Hosoya et al., 1983; Sakurada et al., 1988), semiempirical molecular orbital methods were used to calculate the electronic states of donor molecules. In the present study, we employed an ab initio method for the calculation of electronic states of substrates mentioned above, and the quantum chemical indices obtained were subjected to examination for the correlation with the values for log k_4 which are related to the activation energy of the reaction.

Previously, Bordeleau and Bartha (1972) suggested that the enhancement of the peroxidase-catalyzed oxidation of phenols and anilines upon introduction of electron-releasing substituents may be related to a possible increase in the electron density on oxygen or nitrogen atoms. Our present quantum chemical calculation for phenols and anilines, however, showed that the net charge on oxygen atoms of phenols and on nitrogen atoms of aromatic amines was practically unchanged by substituted groups. This fact, together with no correlation between log k_4 and the net charge on these atoms or carbon atoms, argues against the view that the rate of oxidation by compound II is correlated to the net charge on any atoms.

Instead, high correlations were found between log k_4 and HOMO energy both in phenols and in aromatic amines, in agreement with our previous results (Hosoya et al., 1983). It is noteworthy that the electrons in HOMO were distributed only in $2p\pi$ orbitals and were especially abundant on oxygen or nitrogen atoms and para carbon atoms as mentioned above. These facts indicate that the rate of oxidation by compound II depends on the ease with which a π electron is released from the substrates.

Dunford and collaborators reported that Hammett's rules apply to the oxidation of para- and meta-substituted phenols and anilines by compound I (Job & Dunford, 1976) and compound II (Dunford & Adeniran, 1986). The present results on the oxidation of aromatic substrates by compound II also fitted well into the Hammett equation. Our previous negative results (Hosoya et al., 1983) in applying the Hammett equation to the rate of oxidation of anilines catalyzed by a peroxidase in fungi may be due to the lack of accuracy in the values of oxidation rate obtained by Bordeleau and Bartha (1972) and due to the quantum chemical calculation by a semiempirical method.

Henri-Rousseau and Texier (1978) demonstrated that there is a negative correlation between Hammett's σ value and the HOMO energy levels which were estimated on the basis of the ionization potential and absorption spectrum. This was confirmed as shown in Figure 3, where the HOMO energy

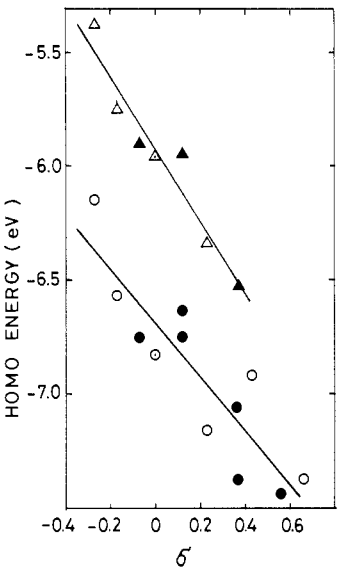


FIGURE 3: Relationship between the HOMO energy levels and Hammett's σ values. (⊙) Phenol; (○) parasubstituted phenols; (●) meta-substituted phenols. (▲) Aniline; (△) para-substituted anilines; (▲) meta-substituted anilines. $r = -0.890$ ($n = 12$) for phenols and $r = -0.961$ ($n = 7$) for anilines.

levels calculated by the ab initio molecular orbital method are plotted against σ values for substituents. Thus, it is considered that a negative correlation between the value and log k_4 has been obtained in the present case. A similar relationship was observed between LUMO and σ values (data not shown).

As described already, the reaction constant (ρ) in the Hammett equation obtained in the present study was −5.75 for oxidation of anilines and −3.78 for oxidation of phenols. These are in agreement with the values for phenols obtained by Dunford and Adeniran (1986), −4.6. According to Hammett (1940), ρ values in many reactions are range between −3.69 and +3.99, depending on the nature and conditions of the reaction: examples with high negative ρ values are reactions between (i) anilines and dinitrochloronaphthalene, −3.69 (Singh & Peacock, 1936), (ii) anilines and dinitrochlorobenzene, −3.19 (Van Opstall, 1933), and (iii) dimethylanilines and trinitrophenol, −2.90 (Hertel & Dressel, 1933). In addition, the following reactions were found to have a high negative ρ value, −5.93 (Bird & Ingold, 1938; Jaffe, 1953):

$$pX\text{–}ArH + NO_2^+ \rightarrow pX\text{–}Ar\text{–}NO_2 + H^+ \qquad (6)$$

These reactions with high negative ρ values are common in that the introduction of electron-releasing substituents enhances the rate of reaction, resembling the present enzymic reaction. In addition, para, meta, and ortho substituents produced nearly the same effect upon the rate of oxidation of phenols and anilines by compound II (Figure 2). These facts are compatible with the view that the effect of substituents on the oxidation rate is related to the step of one electron transfer from the substrate to the heme iron.

However, removing one electron from phenols or anilines to compound II would generate a positive charge in the substrate molecule. If this is the case, the reaction would follow the Okamoto–Brown equation

$$\log k_X/k_H = \rho\sigma^+ \qquad (7)$$

where σ^+ is Okamoto–Brown's substituent constant (Okamoto & Brown, 1957; Brown & Okamoto, 1958). However, our data did not fit the equation, in agreement with the case of oxidation by compound I (Job & Dunford, 1976). Further-

more, the initial products from phenols were reported to the phenoxy radicals (Shiga & Imaizumi, 1978). These facts lead to the supposition that the electron transfer is accompanied by simultaneous loss of a proton from the substrate, avoiding the formation of a positive charge. Since the step expressed by eq 3 was found, by spectrophotometric titration, to consume one proton from the medium (Yamada & Yamazaki, 1974), eq 3 may be expressed more clearly as a whole as

$$AH_2 + H^+ + PoFe^{IV}{=}O \rightarrow AH^\bullet + H_2O + PoFe^{III} \quad (8)$$

where Po stands for protoporphyrin IX. The amount of energy needed to release a proton from OH of phenols may be different from that from NH of anilines. This may be related to the finding that two straight lines with similar slopes were separated by about 0.81 eV as shown in Figure 2.

The dissociation constants of several phenols and anilines to HRP were previously obtained on the basis of optical difference spectra (Paul & Ohlsson, 1979; Hosoya et al., 1989). These values fell in a relatively narrow range, about 3–10 and 10–20 mM, respectively, and no significant correlation was found with log k_4. Thus, the difference in the affinity of donors to HRP is considered to cause little effect on the rate of reaction. Since we are examining a series of monosubstituted phenols and anilines, contribution of the entropy factor for the free energy charge in the reaction is considered not to change significantly among these substrates.

Correlation of HOMO energy levels of organic compounds with their standard redox potentials was reported in the case of nonenzymic oxidation–reduction such as reactions between benzenediols and tris(1,10-phenanthroline)iron(III) (Kimura et al., 1981, 1982; Yamabe et al, 1981). In these studies, the standard redox potentials of the free radicals were estimated from kinetic data by the application of the Marcus theory (Marcus, 1968). Similar correlation between the HOMO energy levels and the standard redox potential of the second substrates is also expected in the present enzyme reactions, but it is difficult at the present stage to calculate accurate standard redox potentials from the kinetic data by the Marcus theory since the enzyme reaction contains more complicated systems.

Therefore, we attempt here only to furnish a qualitative explanation for the results of the present reaction with an adiabatic diagram (Marcus & Sutin, 1985). In Figure 4, R refers to reactants (the left side of eq 8) plus surrounding medium and P denotes products (the right side of eq 8) plus surrounding medium, taking as examples two phenol derivatives [$(AH_2)_{O,i}$ and $(AH_2)_{O,ii}$] in Figure 4a and two aniline derivatives [$(AH_2)_{N,i}$ and $(AH_2)_{N,ii}$] in Figure 4b. The ordinate shows the free energy of the system, but the difference between substrates is considered to be mainly due to that in the electronic energy. At position B, it is expected that the substrate may bind to the enzyme to make it possible to diminish the activation energy of electron transfer through the interaction of R with P (Marcus & Sutin, 1985) or that of HOMO with LUMO (Henri-Rousseau & Texier, 1978; Mulliken & Person, 1969).

In the case of phenols, the following equation is obtained from Figure 4a and eq 8

$$\Delta E_{O,ii} - \Delta E_{O,i} = E_{(AH\cdot)O,ii} - E_{(AH_2)O,ii} - (E_{(AH\cdot)O,i} - E_{(AH_2)O,i}) \quad (9)$$

where $\Delta E_{O,i}$ and $\Delta E_{O,ii}$ are differences in free energies of P and R as shown in Figure 4a and $E_{(AH\cdot)O,ii}$ etc. are electronic energies of respective compounds. Ab initio calculation showed that total energies of radical forms are smaller approximately

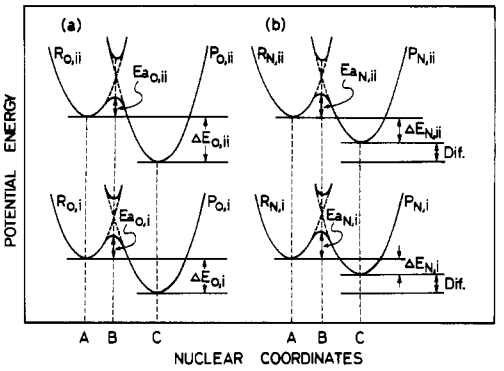


FIGURE 4: Profile of many-dimensional potential surface of reactants plus surrounding medium (R) and that of products plus surrounding medium (P) in the reaction between donors and HRP compound II ($PoFe^{IV}{=}O + H^+ + AH_2 \rightarrow PoFe^{III} + H_2O + AH^\bullet$). A and C denote nuclear conordinates for an equilibrium configuration of reactants and for products, respectively, and B denotes nuclear configuration at the intersection of the two free-energy surface. Hydrogen donors used are (a) two phenols [$(AH_2)_{O,i}$ and $(AH_2)_{O,ii}$] and (b) two anilines [$(AH_2)_{N,i}$ and $(AH_2)_{N,ii}$]. ΔE denotes the difference between energies of P and R, and E_a indicates the activation energy of each reaction. Dif. stands for the difference between ΔE of phenols and ΔE of anilines. For explanation of the figure, see the text.

by the HOMO energy of corresponding donors than those of donors (data not shown). Thus

$$\Delta E_{O,ii} - \Delta E_{O,i} = -(HOMO_{O,ii} - HOMO_{O,i}) \quad (10)$$

where $HOMO_{O,i}$ and $HOMO_{O,ii}$ ($HOMO_{O,ii} > HOMO_{O,i}$) denote the HOMO energies of two of a series of phenol derivatives. On the other hand, the following equation is obtained from the results shown in Figure 1

$$HOMO_{O,ii} - HOMO_{O,i} = \eta\{(\log k_4)_{O,ii} - (\log k_4)_{O,i}\} = -\frac{\eta \times 0.435}{RT}(E_{a,O,ii} - E_{a,O,i}) \quad (11)$$

where η is the slope of the straight line in Figure 1 (0.26 eV for the full line of phenols) and $E_{a,O,i}$ and $E_{a,O,ii}$ are the activation energies of the reaction between compound II and phenols. If it is assumed that the entropy factors of a series of compounds are almost similar to each other as described already, combination of eqs 10 and 11 results in

$$\Delta E_{O,ii} - \Delta E_{O,i} = -(HOMO_{O,ii} - HOMO_{O,i}) = 4.43(E_{a,O,ii} - E_{a,O,i}) \quad (12)$$

This indicates that when two phenol derivatives are compared, the difference in the activation energy is almost equal to one-fifth of the difference in the HOMO energy and that the higher the HOMO energy level of the substrate, the larger the value of $|\Delta E|$ and the smaller the activation energy, E_a.

As for aniline derivatives, similar consideration with the data shown in Figure 1 gives the equation

$$\Delta E_{N,ii} - \Delta E_{N,i} = -(HOMO_{N,ii} - HOMO_{N,i}) = 4.51(E_{a,N,ii} - E_{a,N,i}) \quad (13)$$

thus being very similar to that of phenols. Figure 4b depicts the free energy surface when the energies of R containing $(AH_2)_{N,i}$ and $(AH_2)_{N,ii}$ are almost similar to those with $(AH_2)_{O,i}$ and $(AH_2)_{O,ii}$, respectively. As described under Results, the rate of reaction in anilines is about $^1/_{1000}$ as small as that of phenols with similar levels of HOMO energy (Figure 1), and $E_{a,N,i}$ and $E_{a,N,ii}$ should be larger than $E_{a,O,i}$ and $E_{a,O,ii}$, respectively. Such a difference in the activation energy between phenols and anilines may be explained if the upward

shift of P occurs as shown in Figure 4b, probably due to differences in the bond energy of O–H and N–H, solvent effect of the heteroatoms, the mode of hydrogen binding to the enzyme (Paul & Ohlsson, 1978; Sakurada et al., 1986), and so on.

The above discussion is concerned with the oxidation of substituted phenols and anilines by compound II. The circumstances are, however, similar to the oxidation by compound I as shown in Figure 2. The only difference is that the rate of reaction between the substrate and compound I is much higher than in the case of compound II. This may be related to the fact that compound I has two oxidation equivalents, one in heme iron and the other in the form of cation on porphyrin (Dolphin et al., 1971).

In conclusion, it was found that logarithms of k_4 of monosubstituted phenols and anilines correlated well with HOMO energy levels of these compounds calculated by an ab initio molecular orbital method and that the HOMO energy levels showed a linear relationship with Hammet σ values of substituents with negative slope. Analysis of these correlations suggested that (i) one electron transfer from the substrates to the heme iron may occur from the $2p\pi$ orbital of HOMO of the substrates; (ii) the height of barrier of electron transfer (activation energy) may be a function of the gap between the energy level of the reactants and that of the products, which leads to a linear relationship between HOMO energy and log k_4 of donors, and (iii) the difference in the activation energy of anilines and phenols may be due to concerted proton release from substrates. Further studies are needed to elucidate quantitatively the mechanism of the electron transfer by finding out the configuration of the enzyme–substrate complex.

Acknowledgments

We are grateful to Drs. K. Hiromi, H. Nakatani, and Y. Morita (Kyoto University), Drs. C. Nagata and M. Aida (National Cancer Center Institute), and Dr. J. Ohtsuka (Science University of Tokyo) for helpful discussions. We thank the Computer Center, Institute for Molecular Science, Okazaki National Research Institutes, for the use of HITAC M-680H.

References

Aibara, S., Yamashita, H., Mori, E., Kato, M., & Morita, Y. (1982) *J. Biochem. (Tokyo) 92*, 531–539.

Binkley, J. S., Whiteside, R. A., Raghavachari, K., Seeger, R., DeFrees, D. J., Schlegel, H. B., Frisch, M. J., Pople, J. A., & Kahn, L. R. (1982) GAUSSIAN 82, Release A, Carnegie-Mellon University, Pittsburgh, PA.

Bird, M. L., & Ingold, C. K. (1938) *J. Chem. Soc.*, 918–929.

Bordeleau, L. M., & Bartha, R. (1972) *Can. J. Microbiol. 18*, 1873–1882.

Brown. H. C., & Okamoto, Y. (1958) *J. Am. Chem. Soc. 80*, 4979–4987.

Chance, B. (1951) *Enzymes 2*, Part 1, 428–453.

Dolphin, D., Forman, A., Borg, D. C., Fajer, J., & Felton, R. H. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 614–618.

Dunford, H. B., & Stillman, J. S. (1976) *Coord. Chem. Rev. 19*, 187–251.

Dunford, H. B., & Adeniran, A. J. (1986) *Arch. Biochem. Biophys. 251*, 536–542.

Exner, O. (1972) in *Advances in Linear Free Energy Relationship* (Chapman, N. B., & Shorter, J., Eds.) pp 1–70, Plenum, New York.

Fukui, K. (1970) *Theory of Orientation and Stereoselection*, Springer-Verlag, New York.

George, P. (1952) *Nature (London) 169*, 612–613.

Hammett, L. P. (1940) *Physical Organic Chemistry*, pp 1–228, McGraw-Hill, New York.

Henri-Rousseau, O., & Texier, F. (1978) *J. Chem. Educ. 55*, 437–441.

Hertel, K., & Dressel, J. (1983) *Z. Phys. Chem. B23*, 281–290.

Hiromi, K. (1979) *Kinetics of Fast Enzyme Reaction: Theory and Practice*, Kodansha, Tokyo.

Hosoya, T., Fujii, T., & Ogawa, S. (1983) *J. Theor. Biol. 100*, 283–292.

Hosoya, T., Sakurada, J., Kurokawa, C., Toyoda, R., & Nakamura, S. (1989) *Biochemistry 28*, 2639–2644.

Jaffe, H. H. (1953) *Chem. Rev. 53*, 191–261.

Job, D., & Dunford, H. B. (1976) *Eur. J. Biochem. 66*, 607–614.

Kato, H., Aibara, S., Morita, Y., Nakatani, H., & Hiromi, K. (1984) *J. Biochem. (Tokyo) 95*, 861–870.

Kimura, M., Yamabe, S., & Minato, T. (1981) *Bull. Chem. Soc. Jpn. 54*, 1699–1703.

Kimura, M., Yamamoto, M., & Yamabe, S. (1982) *J. Chem. Soc., Dalton Trans.*, 423–424.

Marcus, R. A. (1968) *J. Phys. Chem. 72*, 891–899.

Marcus, R. A., & Sutin, N. (1985) *Biochim. Biophys. Acta 811*, 265–322.

Marklund, S., Ohlson, P.-I., Opara, A., & Paul, K.-J. (1974) *Biochim. Biophys. Acta 350*, 304–313.

Mulliken, R. S., & Person, W. B. (1969) in *Molecular Complexes, A Lecture and Reprint Volume*, Wiley Interscience, New York.

Okamoto, Y., & Brown, H. C. (1957) *J. Org. Chem. 22*, 485–494.

Paul, K.-G., & Ohlsson, P.-I. (1978) *Acta Chem. Scand. B32*, 395–404.

Poulos, T. L., & Kraut, J. (1980) *J. Biol. Chem. 255*, 10322–10330.

Sakurada, J., Takahashi, S., & Hosoya, T. (1986) *J. Biol. Chem. 261*, 9657–9662.

Sakurada, J., Aida, M., Nagata, C., & Hosoya, T. (1988) *J. Biol. Phys. 16*, 17–23.

Shiga, T., & Imaizumi, K. (1978) *Arch. Biochem. Biophys. 167*, 469–479.

Singh, A., & Peacock, D. H. (1936) *J. Phys. Chem. 40*, 669–677.

Van Opstall, H. J. (1933) *Recl. Trav. Chim. Pays-Bas 52*, 901–911.

Yamabe, S., Minato, T., & Kimura, M. (1981) *J. Phys. Chem. 85*, 3510–3513.

Yamada, H., & Yamazaki, I. (1974) *Arch. Biochem. Biophys. 165*, 728–738.

Yamazaki, I., & Yokota, K. (1973) *Mol. Cell. Biochem. 2*, 39–52.