Rate Constants for Reaction of Hydroxyl Radicals with Water-Soluble Porphyrins and Metalloporphyrins

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To determine the rate constants for the reaction of hydroxyl radicals with water-soluble porphyrins and their metalloporphyrins, tetrakis(4-sulfophenyl)porphine (TPPS), tetrakis(5-sulfothienyl)porphine (T(5-ST)P), tetrakis(4-*N*-methylpyridyl)porphine (TMPyP) and tetrakis(4-*N*-trimethylamino-phenyl)porphine (TTMAP), and Mn(III), Fe(III), Co(III) and Cu(II) complexes of TPPS and TMPyP, the γ-irradiation method, in which the fluorescence of hydroxybenzoates produced through reaction of benzoate with hydroxyl radicals generated by water-radiolysis is measured, were tried. However, since the porphyrins and metalloporphyrins show the characteristic absorption spectra in the visible region, the fluorescence of hydroxybenzoate products was disturbed by the colors. To separate the hydroxybenzoates from the colored solute, the products were extracted into ether under an acidic condition. The hydroxybenzoates were extracted thoroughly into the ether layer from an aqueous layer of less than pH 3 ; the residue was redissolved in phosphate buffer, pH 7.5; the solution was used to determine the rate constants. The rate constants for anionic porphyrins, TPPS, T(5-ST)P, were in the order of 10^9, and the constants for cationic porphyrins, TMPyP, TTMAP, in the order of 10^{10}. Metal complexes of TPPS and TMPyP showed the rate constants of 6.9 to 11.4×10^9 M^{-1} s^{-1} and of 3.2 to 19.7×10^9 M^{-1} s^{-1}, respectively.

Key words rate constant; hydroxyl radical; water-soluble porphyrin; metalloporphyrin; γ-irradiation; hydroxybenzoate

In the active oxygen species, hydroxyl radicals are highly active radicals that react rapidly with most organic and biological substances. Rate constants for their substances have therefore been determined using pulse-radiolysis and various other methods. We recently reported the γ-irradiation method using competitive reaction between benzoate and scavenger for hydroxyl radicals.[1] The rate constants determined by our method were similar to those measured by pulse-radiolysis[2] in which the rate constants obtained are known to be the most accurate among various methods.

Natural metal complexes of porphyrins, iron complexes in hemoproteins and a cobalt complex in Vitamin B_{12}, *etc.* exist in many tissues and exert their important biological activities. Cationic manganese- and iron-porphyrins have been found to be very active for DNA cleavage in the presence of molecular oxygen and a reducing agent.[3] In addition, we have demonstrated that the ion-exchange resins modified with metalloporphyrins exhibit catalase-like,[4] peroxidase-like[5] and uricase-like[6] activities. However, the rate constants for the reaction of these metalloporphyrins with hydroxyl radicals under a physiological condition have not been determined. Accordingly, we tried to examine the reactivity of the metalloporphyrins and the original porphyrins with hydroxyl radicals under physiological conditions.

In our γ-irradiation method, rate constants can be measured using the fluorescence intensity of hydroxybenzoates produced through the reaction of benzoate with hydroxyl radicals. The induced fluorescence is very strong. Porphyrins and metalloporphyrins, however, interfere with the measurement since they have characteristic absorption spectra in the visible region. To determine rate constants for the water-soluble porphyrins and metalloporphyrins, therefore, hydroxybenzoate products must be separated from the colored solute. In this work, we extracted the induced hydroxybenzoates into ether to measure freely the fluorescence intensity. Using the modified γ-irradiation method including the extraction pathway, the rate constants for the water-soluble porphyrins and their metalloporphyrins presented here were measured.

Experimental

Materials Sodium benzoate and 2-hydroxybenzoate (2-HOBZ) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Tetrakis(4-sulfophenyl)porphine (TPPS) and tetrakis(4-*N*-methylpyridyl)porphine (TMPyP) tetra(*p*-toluenesulfonate) were obtained from Tokyo Kasei Co. (Tokyo, Japan). Tetrakis(5-sulfothienyl)porphine (T(5-ST)P) and tetrakis(4-*N*-trimethylaminomethyl)porphine (TTMAP) were from Dojindo Laboratories (Kumamoto, Japan). TPPS and T(5-ST)P were used without further purification to determine rate constants and preparation of metalloporphyrins. TMPyP and TTMAP were used after removal of *p*-toluenesulfonate by anion-exchange resin. All other chemicals used were of the highest purity available. Deionized water (18 MΩ) generated from a Millipore Milli Q purification system was used in all experiments. All reaction mixtures were prepared in Chlex 100-treated Dulbecco phosphate-buffered saline solution without calcium or magnesium ions, pH 7.5 (PBS).[7]

Preparation of Metalloporphyrins Aqueous solutions of metal complexes of TPPS and TMPyP were prepared as described previously.[6] The absorption spectra of the solutions of metalloporphyrins agreed with those reported in the literature.[8] The concentrations of metalloporphyrins were determined using their molar extinction coefficients.

Irradiation One-ml volume of N_2O-saturated 0.2 mM benzoate solution containing various concentrations (0—0.24 mM) of porphyrin or metalloporphyrin was irradiated with a ^{137}Cs-source as done in a previous work.[1]

Measurement of Hydroxybenzoates After irradiation, 200 μl of 0.1 M HCl was added to the benzoate solution. To the acidic solution was then added 1.5 ml cold ether, the mixture was vortexed for 30 s, and was left standing for 10 min in ice. From the ether layer was collected 1.0-ml volume, which was left standing for 20 min in a water bath at 37 °C, and the solvent was then thoroughly removed under a stream of N_2, leaving hydroxybenzoates. The residue was dissolved in 3.0 ml PBS and then the fluorescence intensity of hydroxybenzoates was measured at 407 nm emission after excitation at 305 nm. At least five separate experiments were performed in duplicate.

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Calculation of Rate Constants Rate constants for reaction of hydroxyl radicals with porphyrins and metalloporphyrins (k_S) were calculated using the following equations[1]:

$$\frac{1}{[BZOH]} = \frac{1}{[BZOH]_0} + \frac{k_S[S]}{k_{BZ}[BZ][BZOH]_0} \quad (1)$$

In Eq. 1, a plot of 1/[BZOH] against [S] should give a straight line of slope m with an intercept on the y-axis a

$$m = \frac{k_S}{k_{BZ}[BZ][BZOH]_0} \quad (2)$$

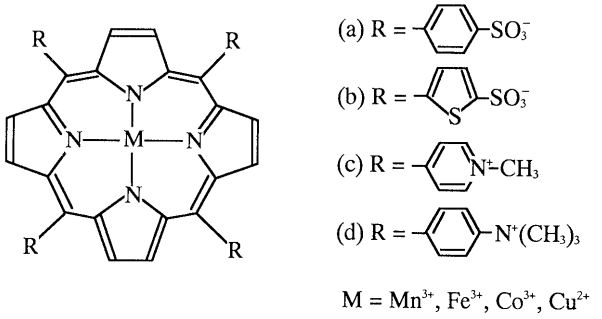
$$a = -\frac{1}{[BZOH]_0} \quad (3)$$

$$k_S = \frac{mk_{BZ}[BZ]}{a} \quad (4)$$

[BZOH] represents the concentration of hydroxybenzoates induced; $[BZOH]_0$, the concentration of hydroxybenzoates induced in the absence of porphyrins or metalloporphyrin; [BZ], 0.2 mM; [S], the concentration of porphyrin or metalloporphyrin used; k_{BZ}, 5.9×10^9 $M^{-1} s^{-1}$. [BZOH] and $[BZOH]_0$ were previously[1] calculated using the concentration of 2-HOBZ induced because 98% of total fluorescence intensity induced was ascribed to 2-HOBZ. In this work, the total fluorescence intensity from hydroxybenzoates induced was used for the determination of rate constants, since the two rate constants calculated using the concentration of 2-HOBZ and the total intensity were similar.

Results and Discussion

Porphyrins and their metalloporphyrins used are illustrated in Fig. 1. The aqueous solutions show characteristic colors, which interfere with the measurement of the fluorescence of hydroxybenzoates produced. In the porphyrins and their metalloporphyrins, the Soret band, an intense absorption band at about 400 nm, has a very high molar extinction coefficient of the order of 1 to 5×10^5. The products, therefore, must be completely separated from the irradiated solution containing porphyrin or metalloporphyrin. Benzoate and hydroxybenzoates are extracted into ether under acidic conditions because of the protonation of carboxylate anion ($pK_a \fallingdotseq 3$). Figure 2 shows the extraction of 2-HOBZ into ether from the acidic benzoate solution; 0.1 M HCl of *ca.* 500 μl was added to 1.0 ml of 0.2 mM benzoate PBS solution containing 2 μM 2-HOBZ. The concentration of 2-HOBZ (2 μM) was decided on the basis of amounts of 2-HOBZ induced by γ-irradiation of the 0.2 mM benzoate solution for 1 h.[1] Ether was added to the acidic solution, and the pH was then determined (Fig. 2, inset); the fluorescence intensities of the ether layers were measured as described in Experimental. When 0.1 M HCl of more than 100 μl was added, the fluorescence intensities of the ether layer were constant at the highest level and the pH values of aqueous layer were less than 3. The recovery of 2-HOBZ extracted into the ether layer was determined by adding 200 μl of 0.1 M HCl (Fig. 3). The fluorescence intensities of ether extracts agreed very closely with those of intact PBS solution of 2-HOBZ. In the case of 3-HOBZ, of which the fluorescence intensity corresponds to 2% of the total intensity of hydroxybenzoates produced, similar results were obtained (data not shown). These results show that the fluorescent products were thoroughly extracted into ether by adding 200 μl of 0.1 M HCl and recovered completely. Further, the water-soluble porphyrins and metalloporphyrins used here were entirely unextracted into ether under the < pH 3 conditions. This modified method



(a) tetrakis(4-sulfophenyl)porphine TPPS
(b) tetrakis(5-sulfothienyl)porphine T(5-ST)P
(c) tetrakis(4-*N*-methylpyridyl)porphine TMPyP
(d) tetrakis(4-*N*-trimethylaminophenyl)porphine TTMAP

Fig. 1. Structure of Porphyrins and Metalloporphyrins

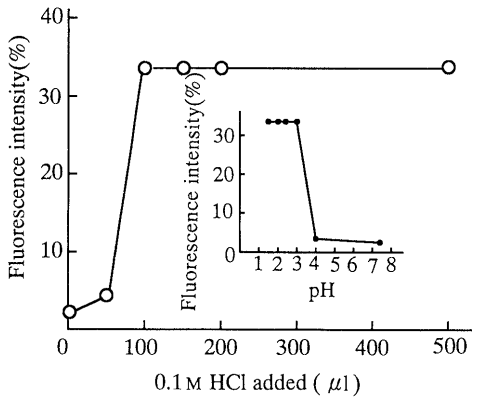


Fig. 2. Extraction of 2-HOBZ into Ether Layer by Addition of 0.1 M HCl

Inset: relationship between the pH values in aqueous layer and the fluorescence intersities of 2-HOBZ in ether layer. The PBS solution (1 ml) contained 0.2 mM benzoate and 2 μM 2-HOBZ. Ether extraction was carried out as described in Experimental.

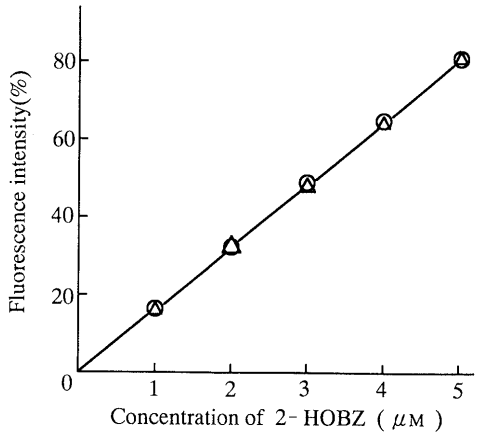


Fig. 3. Recovery of 2-HOBZ Extracted into Ether Layer

The 0.2 mM benzoate solution containing various concentrations of 2-HOBZ (1 ml) was acidified by addition of 200 μl of 0.1 M HCl. The acidic solution was treated with ether as described in Experimental. The circle represents the fluorescence intensity of 2-HOBZ in PBS and the triangle shows tthe intensity of 2-HOBZ extracted into ether.

was used for determination of the rate constant for potassium iodide, and the rate constant obtained was compared with the constant measured by the original method.[1] The two showed similar values. Therefore, the rate constants for the porphyrins and their metallo-

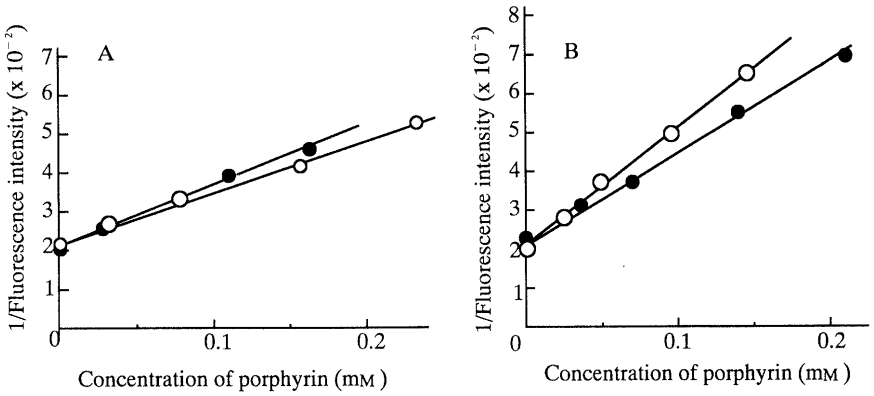


Fig. 4. Reciprocal of the Fluorescence Intensity of 2-HOBZ Produced as a Function of Porphyrin Concentration

A: —○—, TPPS; —●—, T(5−ST)P. B: —○—, TMPyP; —●—, TTMAP. Benzoate solution (0.2 mM) was γ-irradiated for 60 min in the presence of various concentrations of a porphyrin. The irradiated solution was subsequently treated as described in Experimental.

TABLE I. Rate Constants for Reaction of Hydroxyl Radicals with Porphyrins and Metalloporphyrins Determined by Benzoate Hydroxyration

Substance	Rate constant ($\times 10^{-9}$ M^{-1} s^{-1})	Substance	Rate constant ($\times 10^{-9}$ M^{-1} s^{-1})
TPPS	6.8	Mn–TPPS	11.4
T(5-ST)P	9.1	Fe–TPPS	6.9
		Co–TPPS	7.7
TMPyP	17.0	Cu–TPPS	9.1
TTMAP	11.7		
		Mn–TMPyP	8.1
Mn–TPyP	6[a]	Fe–TMPyP	3.2
Hemin	~10[b]	Co–TMPyP	4.2
		Cu–TMPyP	19.7

TPyP: tetrakis(4-pyridyl)porphine. The rate constants were determined in PBS (pH 7.5). The values have deviations of less than ±5%. *a*) From ref. 2. *b*) From ref. 9.

porphyrins were measured using the modified method including the extraction pathway.

Figure 4 shows the results for TPPS, T(5-ST)P, TMPyP and TTMAP measured by the extraction of hydroxybenzoate products into ether. The reciprocal of the fluorescence intensity of the products linearly increased against the concentration of porphyrin added. Such linear plots were obtained in similar experiments with metalloporphyrins. Each rate constant (k_S) was calculated using a slope (m) and an intercept on y-axis (a) of a straight line as described in Experimental. Table I summarizes the results obtained. The rate constants for anionic porphyrins, TPPS and T(5-ST)P, were in the order of 10^9 and the constants for cationic porphyrins, TMPyP and TTMAP, in the order of 10^{10}. For the metal complexes of TPPS , the rate constants were in the range of 6.9 to 11.4×10^9 M^{-1} s^{-1} and higher than that for free TPPS (6.8×10^9 M^{-1} s^{-1}). On the contrary, the rate constants for metal–TMPyP except for Cu–TMPyP were lower than that for TMPyP alone (17.0×10^9 M^{-1} s^{-1}), being in the range of 3.2 to 8.1×10^9 M^{-1} s^{-1}. In previous papers, the rate constant of Mn(III)–TPyP measured at pH 6.8 has been reported to be 6×10^9 M^{-1} s^{-1},[2] and the constant of hemin, Fe(III) complex of protoporphyrin, to be *ca.* 10×10^9 M^{-1} s^{-1}.[9] In this work, the rate constants of Mn–TPPS snd Cu–TMPyP were in the order of 10^{10}, but the other metalloporphyrins were $\sim 10 \times 10^9$ M^{-1} s^{-1}; the reactivity of Mn– and Fe–TPPS and Mn– and Fe–TMPyP with hydroxyl radicals has been determined in the basic solution (pH 11) using a pulse-radiolysis system. The rate constants have been reported to be in the range of 1.5 to 2.4×10^{10} M^{-1} s^{-1}.[10] In the solution of pH 11, the axial ligands of metal complexes are OH^- ions, while the axial ligands at pH 7.5 are water molecules. The difference between the two rate constants obtained under neutral and basic conditions might be related to the axial ligands.

A single test-tube assay using deoxyribose as a detector molecule has been reported for the measurement of the rate constants under physiological conditions.[11] This assay is not applicable to colored substances since a pink chromogen, formed through the reaction of the initial products formed by attack of hydroxyl radicals on deoxyribose with thiobarbituric acid, is colorimetrically determined. The modified γ-irradiation method including the extraction pathway was unaffected by colored substances such as the water-soluble porphyrins and metalloporphyrins. The method described here thus allowed us to measure the rate constants for the colored substances.

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