Effect of Substituted Aryl Group in Water-soluble Porphyrins on 2-Aminofluorene Activation in Ames Assay

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Chemical models for cytochrome P450, consisting of an iron porphyrin complex and an oxidant, have been used as substitutes for the S-9 mix for detecting mutagenicity of promutagens in the Ames assay. In this study, we developed optimized procedures for the Ames mutation assay using a water-insoluble 5,10,15,20-tetrakis(pentafluorophenyl)porphyrinatoiron(III) chloride (F_5P) or a water-soluble 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrinatoiron(III) (4-MPy) plus *tert*-butyl hydroperoxide (*t*-BuOOH) as a chemical model to determine 2-aminofluorene (AF) mutagenicity. The model system including the water-insoluble F_5P plus *t*-BuOOH demonstrated higher AF mutagenicity when the tester strain was added following the incubation period of the reaction mixture. In contrast, in the system including the water-soluble 4-MPy plus *t*-BuOOH, the activity of AF mutagenicity was highest when the tester strain was added to the reaction mixture prior to incubation. It is thus possible to detect short-lived mutagenic metabolites by the latter procedure. AF mutagenicity was compared among diverse water-soluble iron porphyrins plus *t*-BuOOH. The results showed that a cationic 4-MPy/*t*-BuOOH had the highest capacity for mutagenic activation of AF among the chemical models tested.

Key words —— iron porphyrin, chemical model, cytochrome P450, metabolic activation, aromatic amine

INTRODUCTION

The Ames assay is used widely as an initial screen to determine the mutagenic potential of chemicals and drugs.[1] Some carcinogenic chemicals, such as *N*-nitrosamines or heterocyclic amines, are biologically inactive unless they are metabolized to active forms. Since bacteria do not have this metabolic capability, a mammalian-derived exogenous activation system needs to be added to the reaction mixture with the test chemicals and the bacteria. The metabolic activation system usually consists of a rat liver S-9 microsomal fraction in the presence of a NADPH generator. The S-9 mix includes many kinds of cytochrome P450[2] containing iron porphyrin active sites capable of metabolizing a large number of these chemicals to reactive forms.[3] Since the cytochrome P450/NADPH reaction can be mimicked by iron tetraphenylporphyrin in the presence of iodosylbenzene,[4] we have applied chemical models for cytochrome P450, consisting of a porphyrin iron complex and an oxidant to the Ames test. In contrast to the S-9 mix, this chemical model has the advantage lacking substrate and species specificity, the latter due to the non-inclusion of apoproteins. Furthermore, the chemical model is an alternative to the use of experimental animals in primary screening. We have previously reported a chemical model with water-insoluble iron porphyrin 5,10,15,20-tetrakis(pentafluorophenyl)porphyrinatoiron(III) chloride (F_5P) and an oxidant, activated 2-aminofluorene (AF), 2-acetylaminofluorene, benzo[*a*]pyrene, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) and *N*-nitrosodialkylamines.[5,6] In this study, we optimized the procedures for the mutation assay using a water-soluble or a water-insoluble porphyrin iron complex plus an oxidant by using AF mutagenicity in the modified Ames assay. In addition, we investigated the effect of the structure of water-soluble porphyrins, which have fluoro groups or a methyl group at the *ortho* position in arylporphyrins, on AF mutagenicity in the presence of *tert*-butyl hydroperoxide (*t*-BuOOH).

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MATERIALS AND METHODS

Materials —— AF (CAS No. 153-78-6), F_5P (CAS No. 36965-71-6) and *t*-BuOOH (CAS No. 75-91-2) were from Aldrich Chem. Co. (Milwaukee, WI, U.S.A.). 5,10,15,20-Tetrakis(1-methylpyridinium-4-yl)porphine *p*-toluenesulfonate (CAS No. 36951-72-1) and 5,10,15,20-tetrakis(4-sulfonatophenyl)-porphine (CAS No. 35218-75-8) were purchased from Tokyo Kasei (Tokyo, Japan). 5,10,15,20-Tetrakis(1-methylpyridinium-2-yl)porphine tetrachloride (CAS No. 129051-18-9), 5,10,15,20-tetrakis(4-*N*,*N*,*N*-trimethylanilinium-4-yl)porphyrinatoiron(III) chloride (CAS No. 161776-18-7), 5,10,15,20-tetrakis(2,3,5,6-tetrafluorophenyl-4-*N*,*N*,*N*-trimethylanilinium-4-yl)porphyrinatoiron(III) CF_3SO_3 salt (CAS No. 196715-03-4) and 5,10,15,20-tetrakis(2,6-difluoro-4-sulfonatophenyl)porphyrin sodium salt were from Mid-Century Chemicals (Chicago, IL, U.S.A.). 5,10,15,20-Tetrakis(1-methylpyridinium-4-yl)porphyrinatoiron-(III) pentachloride (4-MPy),[7] 5,10,15,20-tetrakis-(1-methylpyridinium-2-yl)porphyrinatoiron(III) pentachloride (2-MPy),[8] 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinatoiron(III) (FeTPPS)[9] and 5,10,15,20-tetrakis(2,6-difluoro-4-sulfonatophenyl)porphyrinatoiron(III) (F_2S)[10] were synthesized as described. Porphyrin purity was determined by ultraviolet spectroscopy. Figure 1 shows the structure of iron porphyrins used in this study.

Bacterial Mutation Assay Using the Chemical Model —— The bacterial mutation assay was based on the Ames test,[1] utilizing a chemical model consisting of iron(III) porphyrin and an oxidant as a substitute for the metabolic activation system.

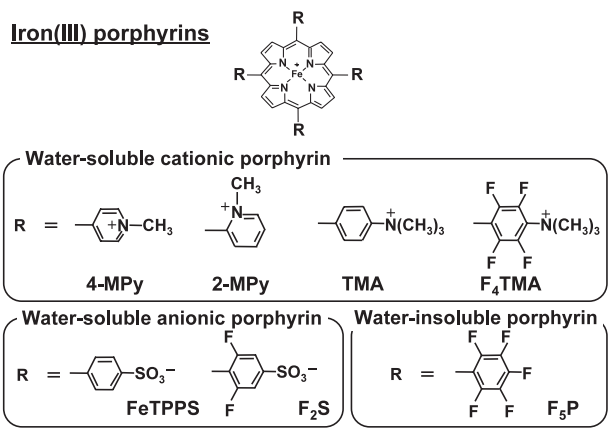

Fig. 1. Structure of Iron Porphyrins Used in this Study

Salmonella typhimurium (*S. typhimurium*) TA1538 was provided by Prof. B. N. Ames, the University of California, Berkeley, CA, U.S.A. Water-soluble porphyrins and *t*-BuOOH were dissolved in 0.1 M sodium phosphate buffer (pH 7.4). Mutagen and F_5P were dissolved in acetonitrile. Concentrations are listed in the figure legends.

Preincubation Assay Involves Exposing a Tester Strain with Reaction Mixture —— Porphyrin (20 μl) and AF (20 μl), both in solution, were mixed, and oxidant solution (20 μl), 0.1 M sodium phosphate buffer (pH 7.4, 0.5 ml), and a culture of tester strains (0.1 ml) were added. The mixture was then incubated for 10 sec, 1, 3, 5, 10, or 20 min at 25°C with shaking (120 strokes/min), and top agar (2 ml) was added. The mixture was then poured onto a minimal-glucose agar plate. After incubation for 44 hr at 37°C, colonies were counted. All plates were prepared in duplicate and the experiments were repeated at least twice. Data are presented as the mean of duplicate determinations.

Preincubation Assay Involves Adding a Tester Strain into a Reaction Mixture after Incubation —— An aliquot of F_5P (20 μl) and AF (20 μl) in solution were mixed, and *t*-BuOOH (20 μl) in 0.1 M sodium phosphate buffer (pH 7.4) was added. The mixture was then incubated for 1, 3, 5, 10 or 20 min at 25°C with shaking (120 strokes/min), and 0.1 M sodium phosphate buffer (pH 7.4, 0.5 ml), a culture of tester strains (0.1 ml), and top agar (2 ml) were added. The following procedures were performed the same as those for the assay described above.

RESULTS

Development of Ames Assay Protocols Using Chemical Models

Experimental procedures for the Ames assay using a chemical model as a substitute for the S-9 mix were developed. AF activation by water-insoluble F_5P or water-soluble 4-MPy plus *t*-BuOOH was compared among the procedures where a tester strain was present or absent from a reaction mixture containing AF with the activation system (Figs. 2 and 3). Figure 2 shows the effect of the reaction period of AF mutagenicity in the presence of each chemical model. AF was mutagenic at all incubation periods, however for each chemical model the highest mutagenic activity occurred at a different incubation period. In this study, the optimal incu-

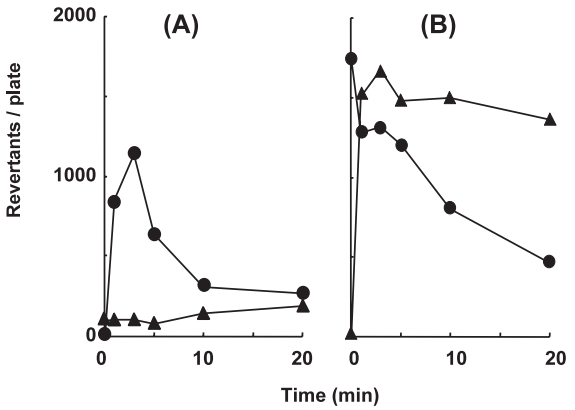

Fig. 2. Effect of Incubation Period on AF Mutagenicity in *S. typhimurium* TA1538

(A) AF mutagenicity in the presence of F_5P and *t*-BuOOH in different methods. Preincubation assay involves exposing a tester strain with reaction mixture (▲) or adding a tester strain into a reaction mixture after incubation period (●). The concentrations consisted of AF (100 nmol per plate), F_5P (50 nmol per plate) and *t*-BuOOH (150 nmol per plate). (B) AF mutagenicity in the presence of 4-MPy and *t*-BuOOH in different methods. Preincubation assay involves exposing a tester strain with reaction mixture (▲) or adding a tester strain into a reaction mixture after incubation period (●). The concentrations consisted of AF (100 nmol per plate), 4-MPy (50 nmol per plate) and *t*-BuOOH (150 nmol per plate).

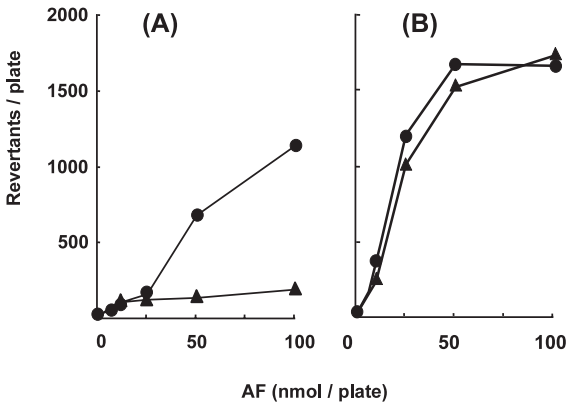

Fig. 3. Effect of Methods on AF Mutagenicity by a Chemical Model

The preincubation assay includes adding a tester strain into a reaction mixture after incubation (●). The preincubation assay includes exposing a tester strain to the reaction mixture (▲). The concentrations consisted of iron(III) porphyrin (50 nmol per plate) and *t*-BuOOH (150 nmol per plate). (A) Mutagenicity of AF by F_5P/*t*-BuOOH. F_5P (●) incubation for 3 min, (▲) incubation for 20 min. (B) Mutagenicity of AF by 4-MPy/*t*-BuOOH. 4-MPy (●) incubation for 3 min, (▲) incubation for 10 sec.

bation period for each of the assay conditions was selected for data presentation.

Figure 3 shows a comparison of the highest AF mutagenic activity with each of the two assay procedures using the respective optimal incubation periods. AF mutagenicity in the presence

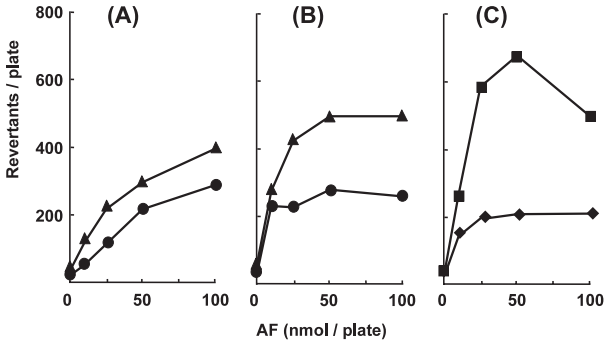

Fig. 4. Effect of Substituted Aryl Group in Water-soluble Porphyrins on AF Mutagenicity

(A) Anionic porphyrins (50 nmol per plate) and *t*-BuOOH (150 nmol per plate). F_2S (▲) incubation for 10 sec, FeTPPS (●) incubation for 10 sec. (B) Cationic porphyrins (10 nmol per plate) and *t*-BuOOH (30 nmol per plate). F_4TMA (▲) incubation for 3 min, TMA (●) incubation for 5 min. (C) Cationic porphyrins (10 nmol per plate) and *t*-BuOOH (30 nmol per plate). 4-MPy (■) incubation for 1 min, 2-MPy (◆) incubation for 1 min.

of the chemical model utilizing the water-insoluble F_5P/*t*-BuOOH demonstrated higher AF mutagenicity when a tester strain was added after incubation as compared to adding tester strain prior to incubation. When water-insoluble F_5P/*t*-BuOOH was used, the highest mutagenic activity was observed by including a 3 min delay between the incubation period and addition of a tester strain. The results indicated that porphyrin solubility was a key factor for AF mutagenicity since F_5P was precipitated during reaction with a tester strain and reaction mixture. AF mutagenicity in both procedures was similar in the presence of a water-soluble 4-MPy/*t*-BuOOH.

The experimental procedures were optimized to include addition of a tester strain after incubation in a water-insoluble F_5P/oxidant system. In contrast, a water-soluble porphyrin/oxidant system was suitable for an assay system in which a tester strain was added to the reaction mixture during the incubation period. Comparison of AF mutagenicity under optimal conditions for 4-MPy or F_5P plus *t*-BuOOH indicated that the cationic porphyrin was more effective than F_5P.

Effect of Aryl Group in Water-soluble Iron(III) Porphyrin on AF Mutagenicity

AF mutagenicity was compared among various water-soluble porphyrins when *t*-BuOOH was used (Fig. 4).

The cellular toxicity of 5,10,15,20-tetrakis(4-*N*,*N*,*N*-trimethylanilinium-4-yl)porphyrinatoiron(III) pentachloride (TMA) and 5,10,15,20-tetrakis(2,3,5,6-tetrafluoro-4-*N*,*N*,*N*-trimethylanilinium-4-yl)por-

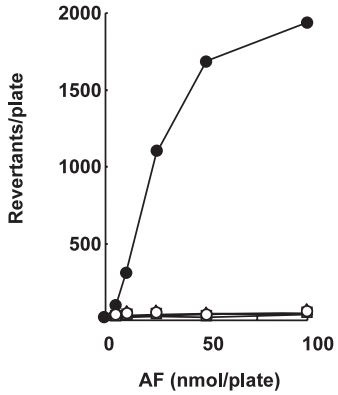

Fig. 5. Mutagenicity of AF in *S. typhimurium* TA1538 under Optimal Conditions

Optimal conditions consisted of 4-MPy (50 nmol per plate) and *t*-BuOOH (150 nmol per plate) in sodium phosphate buffer (pH 7.4) at 3 min. The complete system contained AF, 4-MPy and *t*-BuOOH (●). The control system; without 4-MPy (□), without *t*-BuOOH (△), and AF alone (○). □, △ and ○ were overlapped.

phyrinatoiron(III) pentachloride (F_4TMA) occurred at the same concentration as 4-MPy, indicating that the concentration of the cationic chemical model was five factors less than that of the anionic ones. The potency of AF mutagenicity was observed in the following order: 4-MPy = F_4TMA > 2-MPy = TMA > F_2S > FeTPPS. Figure 5 shows the mutagenicity of the AF mixture under optimal conditions. 4-MPy plus *t*-BuOOH in the absence of AF was not mutagenic, and the reaction mixture was not mutagenic in the absence of either 4-MPy or *t*-BuOOH.

DISCUSSION

In the current study we describe the development of chemical models for cytochrome P450 as an alternative to the S-9 mix in the Ames assay. In this study, these chemical models consisted of either a water-insoluble or a water-soluble iron porphyrin and an oxidant. We optimized the experimental procedures for mutation assays using these chemical models with AF mutagenicity. AF mutagenicity with water-insoluble F_5P/*t*-BuOOH was higher when the tester strain was added following the incubation as compared to the tester strain being present during incubation (Figs. 2 and 3). When water-soluble 4-MPy/*t*-BuOOH was used, AF mutagenicity was similar in both procedures. The F_5P/oxidant system was developed, and was added to a tester strain after activation of AF since it is easy to detect highly unstable intermediate in aqueous solution.[5)] A water-soluble porphyrin plus an oxidant system was developed to expose a tester strain for an incubation period with the chemical model since short-lived mutagenic metabolites have a better chance of reacting with the tester strain in the activation system, thus making it possible to test different mutagenic compounds using the respective procedure. Furthermore, we compared AF mutagenicity among diverse water-soluble iron porphyrins in the presence of *t*-BuOOH since AF activity under optimal conditions increased more by using 4-MPy than by using F_5P (Fig. 4). Aryl porphyrins substituted with fluoro groups at *ortho* positions are known to be stable towards oxidative degradation of porphyrins due to a combination of steric and electron-withdrawing effects that decrease oxidation of the phenyl group in the porphyrin ring.[11)] Mutagenic influence of porphyrin at optimal conditions was in the following order: 4-MPy > F_4TMA > TMA = 2-MPy > F_2S > FeTPPS. The results showed that the chemical model using cationic porphyrin activated AF more than anionic porphyrin. It has been reported that cationic 4-MPy had a lower tendency to aggregate than anionic FeTPPS.[12)] AF mutagenicity in the presence of F_2S or F_4TMA plus *t*-BuOOH was higher than that without the fluoro group, which affected the oxidative active porphyrin stability. In iron porphyrins with the *N*-methylpyridylporphyrins, although the 2-isomer is more resistant to oxidative destruction,[7,8)] AF mutagenicity by 2-MPy plus *t*-BuOOH was lower than that by 4-MPy plus *t*-BuOOH. 2-MPy has a methyl group at the *ortho* position, which is larger than the fluoro group, thus it might be difficult for the mutagen to approach oxoironporphyrin due to steric effects of the methyl group. 4-MPy/*t*-BuOOH showed the highest capacity for AF mutagenic activation among the diverse chemical models. The combination of 4-MPy/*t*-BuOOH oxidized AF to DNA-damaging species since 4-MPy plus *t*-BuOOH in the absence of AF was not mutagenic and the reaction mixture was also not mutagenic in the absence of either 4-MPy or *t*-BuOOH (Fig. 5).

The AF activation is known to be a hydroxylation by cytochrome P450 to *N*-hydroxy-2-aminofluorene, follows by an acetylation by *O*-acetyltranferase as a phase II enzyme.[13)] We have shown that 2-acetylaminofluorene in the presence of water-insoluble F_5P/*t*-BuOOH was oxidized to *N*-hydroxy-2-acetylaminofluorene, and then further to 2-nitro-9-fluorenone, which was mutagenic in *S. typhimurium* TA1538.[14)] On the other hands, we es-

timated that *N*-hydroxylamino compounds derived from AF was mutagenic in the presence of water-soluble 4-MPy/*t*-BuOOH.

The mutagenicity of aromatic amines and amides with the chemical model was lower than that with the S-9 mix,[15] probably because of a lack of phase II enzymes in the chemical models. However 4-MPy/*t*-BuOOH apparently activated aromatic amines to mutagenic species in the present investigation.

In summary, we developed mutation assays using chemical models to replace the S-9 mix. The F_5P chemical model was suitable for adding a tester strain following incubation, whereas a chemical model containing a water-soluble porphyrin was suitable for addition of a tester strain during incubation. We developed 4-MPy/*t*-BuOOH to detect AF mutagenicity in the Ames assay without using the enzymatic activating system.

The results expand the conditions of mutation assay using the model system not only in nonaqueous solution but in aqueous solution.

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