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Reduction of Tertiary Amine *N*-Oxides by Cytochrome P-450

Mechanism of the Stimulatory Effect of Flavins and Methyl Viologen

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SUMMARY

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The stimulatory effect of flavins (riboflavin, FMN, and FAD) and viologens (methyl viologen and benzyl viologen) on the anaerobic reduction of tertiary amine *N*-oxides in liver microsomes was investigated. The reduced flavins or viologens hardly reduced tiaramide *N*-oxide and imipramine *N*-oxide in the absence of microsomes. The stimulatory effect of flavins or viologens was completely abolished under an atmosphere of carbon monoxide. FMN or methyl viologen added to the microsomal suspension was rapidly reduced after the addition of NADPH, probably through NADPH-cytochrome *c* reductase. The reduced FMN or methyl viologen was rapidly reoxidized upon addition of the *N*-oxides, probably through the oxidation-reduction cycle of cytochrome P-450 initiated by the *N*-oxides. Although a xanthine oxidase system (xanthine oxidase plus hypoxanthine) did not appreciably reduce cytochrome P-450, tiaramide *N*-oxide, or imipramine *N*-oxide, reduction of the *N*-oxides was markedly stimulated in the presence of flavins or viologens. FMN or methyl viologen in xanthine oxidase-containing microsomal suspensions was rapidly reduced after the addition of hypoxanthine. The reduced FMN or methyl viologen was rapidly reoxidized upon addition of the tertiary amine *N*-oxides concomitantly with the formation of the corresponding amines. The stimulatory effect of FMN or methyl viologen on the xanthine oxidase-supported *N*-oxide reduction was dependent on the amount of added xanthine oxidase and was completely blocked under an atmosphere of carbon monoxide. The maximum stimulation by FMN and methyl viologen of NADPH-supported tiaramide *N*-oxide reduction was about 10- and 100-fold, respectively. The maximal activity of xanthine oxidase-supported *N*-oxide reductase in the presence of FMN or methyl viologen was about 200 or 6000 nmoles/mg of protein per minute, respectively. Using chemically reduced FMN and methyl viologen as electron donors and tiaramide *N*-oxide as substrate, maximal reduction rates of 500 and 10,000 nmoles/mg of protein per minute were recorded, respectively. Therefore the maximum turnover number of cytochrome P-450 should be more than 12,000/min. These results indicate that the rate-limiting step in the reduction of *N*-oxides is the reduction of cytochrome P-450, and other steps seem to be very fast. It was therefore concluded that the mechanism of flavin- or viologen-induced stimulation of NADPH-dependent *N*-oxide reduction consists of the reduction of flavins or viologens by NADPH-cytochrome *c* reductase and the reduction of cytochrome P-450 by the reduced form of flavins or

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viologens. Experiments with purified cytochrome P-450 and NADPH–cytochrome *c* reductase confirmed this conclusion.

INTRODUCTION

Hepatic cytochrome P-450, a component of the microsomal monooxygenase system, which catalyzes the oxidative metabolism of various xenobiotics, is known as the activating enzyme of molecular oxygen and the substrate binding site. In previous papers we have reported that the reduced form of cytochrome P-450 catalyzes the reduction of tertiary amine *N*-oxides to corresponding tertiary amines (1, 2). The reduction is NADPH-dependent and inhibited by both carbon monoxide and oxygen. Evidence has also been presented that the reduction of tertiary amine *N*-oxides by liver microsomes under a nitrogen atmosphere is markedly stimulated by addition of flavins or methyl viologen (3). The mechanism of stimulation by flavins seemed to be involved in acceleration of the reduction of cytochrome P-450 through the reduced forms of the flavins (3).

This communication reports the mechanism and nature of flavin- and methyl viologen-induced stimulation of tertiary amine *N*-oxide reduction in relation to the reduction rate of cytochrome P-450 by intact hepatic microsomes and a purified cytochrome P-450 system.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats, aged 7 weeks, were used unless otherwise specified. Some animals were treated with phenobarbital (80 mg/kg, intraperitoneally daily for 3 days. Adult male rabbits were treated with phenobarbital (50 mg/kg, intraperitoneally) daily for 5 days. Phenobarbital-treated animals were killed 24 hr after the last injection.

Materials. NADPH (type 1), FAD, FMN, riboflavin, methyl viologen, benzyl viologen, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, cytochrome *c*, hypoxanthine, and xanthine oxidase were purchased from Sigma Chemical Company.

Tiaramide (4-[(5-chloro-2-oxo-3(2*H*)-benzothiazolyl)acetyl]-1-piperazineethanol] and tiaramide *N*-oxide were synthesized in Fujisawa Research Laboratories. Imipramine was donated by Ciba-Geigy. Imipramine *N*-oxide was synthesized by the method of Fishman and Goldenberg (4). Emulgen 913, a polyoxyethylene nonylphenyl ether, was kindly supplied by Kao-Altas Company.

Homogeneous cytochrome P-450 used in the present experiments was prepared from phenobarbital-treated rabbit liver microsomes as described by Imai and Sato (5). NADPH–cytochrome *c* reductase was partially purified from liver microsomes of phenobarbital-treated rats as described by Imai (6). In most experiments the perfused liver was homogenized in 3 volumes of a 1.15% KCl solution, and liver microsomes were prepared as described previously (1).

Assay of tertiary amine N-oxide reductase activity. Tertiary amine *N*-oxide reductase activity of rat liver microsomes was assayed by measuring the formation of tertiary amines. The standard assay medium (2.5 ml) contained 150 μmoles of phosphate buffer (pH 7.4), 5 μmoles of NADPH, 25 μmoles of $MgCl_2$, 25 μmoles of glucose 6-phosphate, 3.0 IU of glucose 6-phosphate dehydrogenase, 2.5 mg of microsomal protein, and the indicated tertiary amine *N*-oxide at a concentration of 1.0 mM. The incubation was carried out anaerobically at 37° for 1–5 min. The anaerobic experiments, under either an N_2 or a CO atmosphere, were carried out in Thunberg vessels. The incubation mixtures were alternately evacuated and flushed with nitrogen or carbon monoxide, which was passed through a deoxygenizer system (7).

In spectrophotometric studies, anaerobic experiments, under an atmosphere of either N_2 or CO, were carried out at 37° in cuvetts equipped with a serum cap by continuous bubbling of the gases. The solutions added to the incubation mixtures, in volumes of 50 μl, had previously been evacuated and flushed with nitrogen or carbon monoxide.

Analytical methods. The formation of tertiary amines was measured using gas chromatography as described previously (1,

2). Cytochrome P-450 was determined from CO difference spectra of dithionite-treated samples as described by Omura and Sato (8). Protein was determined by the method of Lowry *et al.* (9), with bovine serum albumin as standard.

The reduction rates of FMN and methyl viologen were determined with a Hitachi 356 two-wavelength, double-beam spectrophotometer by the decrement in absorbance at 450 nm and the increment in absorbance at 600 nm, respectively, after the addition of NADPH or hypoxanthine to the microsomal suspensions described in the legends to the figures.

RESULTS

Stimulation by flavins and viologens of NADPH-dependent N-oxide reduction by liver microsomes. The addition of riboflavin, FMN, or FAD markedly stimulated the reduction of tiaramide *N*-oxide (Fig. 1). Riboflavin was the most effective, and FAD was the least. These stimulatory effect of flavins were almost completely abolished under an atmosphere of carbon monoxide, as reported in a previous paper (3) (data not shown). Higher concentrations of riboflavin, FMN, and FAD decreased their stimulatory effects. For example, the maximum stimulatory effect of FMN was obtained at about 100 μM, and that of riboflavin at about 500 μM. At 5 mM, however, neither riboflavin nor FMN had any stimulatory effect.

Benzyl viologen and methyl viologen stimulated the reduction of tiaramide *N*-oxide more than 100-fold. The stimulatory effect of methyl viologen increased with increases in its concentration (Fig. 2). These stimulatory effects of the viologens were almost completely abolished under an atmosphere of carbon monooxide, as reported previously (3) (data not shown). The stimulatory effect of benzyl viologen seemed to be saturated at 1 mM.

NADPH-dependent reduction of FMN and its reoxidation by tertiary amine N-oxide in liver microsomes. Kamm and Gillette (10) reported that rat liver microsomes can rapidly reduce added FAD under an atmosphere of nitrogen. After addition of NADPH to the microsomal suspension,

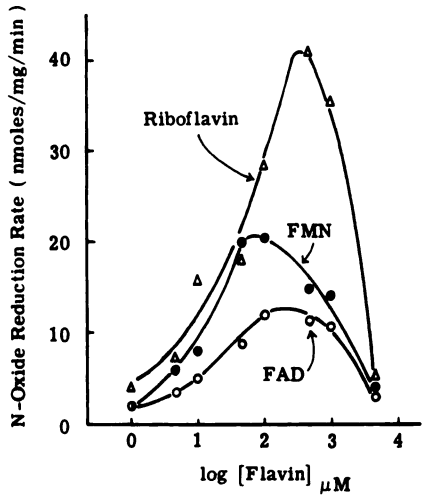

FIG. 1. *Effect of flavin concentration on NADPH-dependent tiaramide N-oxide reduction by liver microsomes*

The incubation mixture (2.5 ml) consisted of 2.5 mg of protein, 5 μmoles of NADPH, 2.5 μmoles of tiaramide *N*-oxide, and other components described in MATERIALS AND METHODS. The incubation was carried out for 5 min anaerobically.

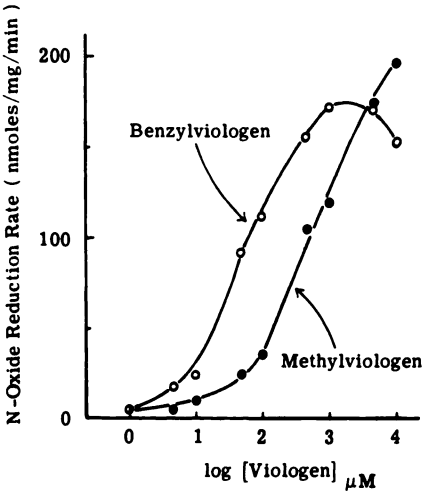

FIG. 2. *Effect of viologen concentration on NADPH-dependent tiaramide N-oxide reduction by liver microsomes*

The incubation mixture (2.5 ml) consisted of 2.5 mg of protein, 5 μmoles of NADPH, 10 μmoles of tiaramide *N*-oxide, and other components described in MATERIALS AND METHODS. The incubation was carried out for 5 min anaerobically.

FMN was rapidly reduced under a nitrogen atmosphere (Fig. 3a). The maximum rate of reduction of FMN was about 16

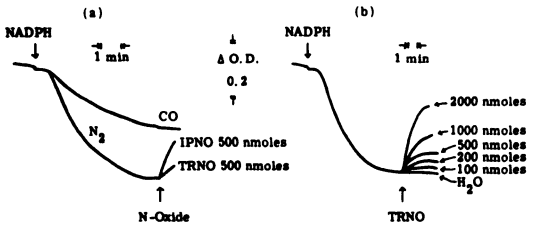

FIG. 3. *NADPH-dependent reduction of FMN and its reoxidation by tertiary amine N-oxides by liver microsomes*

a. Effect of imipramine *N*-oxide and tiaramide *N*-oxide. The incubation mixture (2.4 ml) consisted of 2.5 mg of protein, 125 nmoles of FMN, and other components described in MATERIALS AND METHODS. NADPH (2.5 μmoles), imipramine *N*-oxide (IPNO; 500 nmoles), and tiaramide *N*-oxide (TRNO; 500 nmoles) were added anaerobically at the indicated times. The change in absorbance at 450 nm was recorded under an atmosphere of nitrogen or carbon monoxide. The formation of imipramine and tiaramide was determined 1 min after addition of the *N*-oxides.

b. Effect of various concentrations of tiaramide *N*-oxide. The incubation mixture was the same as in Fig. 3a. Various amounts of tiaramide *N*-oxide were added anaerobically at the indicated time.

nmoles/mg of protein per minute. At the steady state of the oxidation-reduction cycle about 90% of FMN existed in the reduced form. The reduced FMN was rapidly reoxidized by the addition of tertiary amine *N*-oxides. The initial rates of reoxidation of reduced FMN by the addition of imipramine *N*-oxide or tiaramide *N*-oxide were about 31 and 4.8 nmoles/mg of protein per minute, respectively. The rates of formation of imipramine and tiaramide under these conditions were 35 and 5.2 nmoles/mg of protein per minute, respectively. Under a carbon monooxide atmosphere, the change in absorbance at 450 nm due to FMN reduction was affected by the formation of a cytochrome P-450 · CO complex, but the reoxidation of reduced FMN by the addition of tertiary amine *N*-oxides was completely abolished and imipramine and tiaramide were formed at 2% and 3%, respectively, of the rates obtained under a nitrogen atmosphere. These results suggest that imipramine *N*-oxide and tiaramide *N*-oxide react with reduced cytochrome P-450 and initiate the oxidation-reduction cycle of cytochrome P-450. The rate of reduction of the tertiary amine *N*-oxide represents the rate of the oxidation-reduction cycle of cytochrome P-450 (turnover number) in liver microsomes. A 1:1 stoichiometry for NADPH consumption and tertiary amine formation was reported previously (2).

The rate of reoxidation of reduced FMN by tiaramide *N*-oxide was related to the concentration of added *N*-oxide (Fig. 3b). After the addition of a small amount of *N*-oxide, a new steady state of the oxidation-reduction cycle of FMN was reestablished within a short time, whereas after the addition of a large amount of *N*-oxide, reestablishment of a new oxidation-reduction equilibrium for FMN took longer and the steady-state level was found at a high oxidation state. The initial rates of tiaramide *N*-oxide reduction were 3.1, 5.8, 11.8, and 22.2 nmoles/mg of protein per minute on the addition of 200, 500, 1000, and 2000 nmoles of tiaramide *N*-oxide, respectively.

NADPH-dependent reduction of methyl viologen and its reoxidation by tertiary amine N-oxide in liver microsomes. The reduction of methyl viologen by liver microsomes in the presence of NADPH was reported by Bus *et al.* (11). The rate of reduction of methyl viologen was faster than that observed with FMN (Figs. 3a and 4a); a maximum rate of about 47 nmoles/mg of protein per minute was observed. These results confirm the observations of Bus *et al.* (11). However, only about 3% of the methyl viologen was in the reduced form in the steady state. On the addition of tertiary amine *N*-oxides, the reduced methyl viologen was rapidly oxidized (Fig. 4a). The initial rates of reoxidation of reduced methyl viologen on the addition of imipramine *N*-oxide and tiaramide *N*-oxide were about 140 and 79 nmoles/mg of protein per minute, respectively. The rates of formation of imipramine and tiaramide under these conditions were 110 and 93 nmoles/mg of protein per minute, respectively. Under a carbon monoxide atmosphere, the reoxidation of reduced methyl viologen was completely inhibited and the amounts of imipramine and tiaramide formed were about 2% and 4%, respectively, of those obtained under nitrogen. The rate of reoxidation of reduced methyl viologen by tiaramide *N*-oxide was related to the concentration of the added

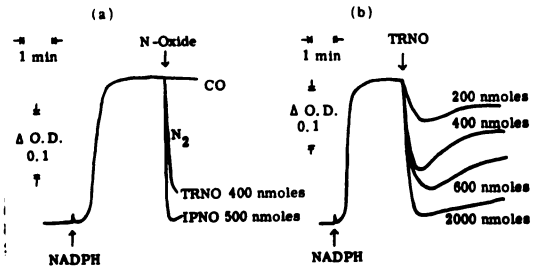

FIG. 4. *NADPH-dependent reduction of methyl viologen and its reoxidation by tertiary amine N-oxides by liver microsomes*

a. Effect of imipramine *N*-oxide and tiaramide *N*-oxide. The incubation mixture (2.4 ml) consisted of 2.5 mg of protein, 2500 nmoles of methyl viologen, and other components described in MATERIALS AND METHODS. NADPH (2.5 μmoles), tiaramide *N*-oxide (TRNO; 400 nmoles), and imipramine *N*-oxide (IPNO; 500 nmoles) were added anaerobically at the indicated times. The change in absorbance at 450 nm was recorded under an atmosphere of nitrogen or carbon monoxide. The formation of tiaramide and imipramine was determined 1 min after addition of the *N*-oxides.

b. Effect of various concentrations of tiaramide *N*-oxide. The incubation mixture was the same as in Fig. 4a. Various amounts of tiaramide *N*-oxide were added anaerobically at the indicated times.

N-oxide (Fig. 4b). After the addition of a small amount of *N*-oxide, a new steady state of oxidation and reduction of methyl viologen was established within a short time.

Xanthine oxidase-supported N-oxide reduction by hepatic cytochrome P-450 in the presence of flavins and viologens. The hypoxanthine-xanthine oxidase system reduced hepatic cytochrome P-450 very slowly, as reported by Ichikawa and Yamano (12), and it also reduced very little tiaramide *N*-oxide. In the presence of flavins, however, the xanthine oxidase system markedly stimulated the reduction of tertiary amine *N*-oxides, probably through the reduction of flavins and cytochrome P-450 (Fig. 5). The reduction of tiaramide *N*-oxide in the absence of flavins was negligible, and the stimulatory effect of flavins increased with their concentration. Riboflavin was the most effective, and FAD was the least. In contrast to their stimulatory effect on NADPH-dependent tiaramide *N*-oxide reduction, high concentrations of flavins did not decrease the stimulatory effect of xanthine oxidase-supported *N*-oxide reduction. At 5 mM, riboflavin stimulated tiaramide *N*-oxide reduction about 100-fold. The addition of benzyl viologen or methyl viologen markedly stimulated the reduction of tiaramide *N*-oxide; approximately 300- and 230-fold stimulation was obtained with 10 mM benzyl viologen and methyl viologen, respectively (Fig. 6). The stimulatory effects of the flavins and viologens on xanthine oxidase-supported tertiary amine *N*-oxide reduction were almost completely abolished under an atmosphere of carbon monoxide (data not shown).

Xanthine oxidase-supported reduction of FMN and its reoxidation by tertiary amine N-oxide in liver microsomes. On the addition of hypoxanthine, FMN was rapidly reduced by xanthine oxidase under a nitrogen atmosphere. The maximum rate of reduction of FMN was about 36 nmoles/min (Fig. 7a). The reduced FMN was rapidly reoxidized on the addition of imipramine *N*-oxide and tiaramide *N*-oxide, with initial reoxidation rates of about 545 and 252 nmoles/mg of protein per minute, respectively, and the corresponding formation of imipramine and tiaramide of 440 and 300 nmoles/mg of protein per minute. Under a

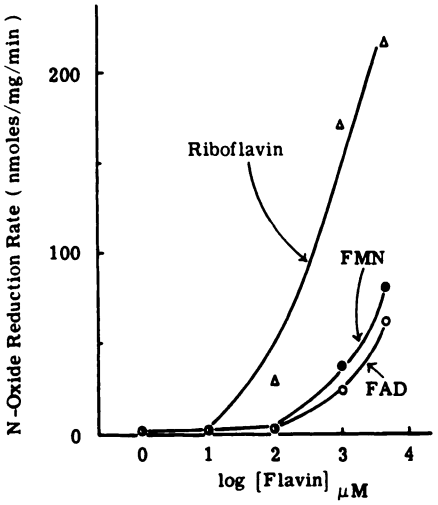

FIG. 5. *Effect of flavin concentration on xanthine oxidase-supported tiaramide N-oxide reduction by liver microsomes*

The incubation mixture (2.5 ml) consisted of 1.25 mg of protein, 10 μmoles of tiaramide *N*-oxide, 0.5 unit of xanthine oxidase, 10 μmoles of hypoxanthine, and 150 μmoles of phosphate buffer, pH 7.4. The incubation was carried out for 5 min anaerobically.

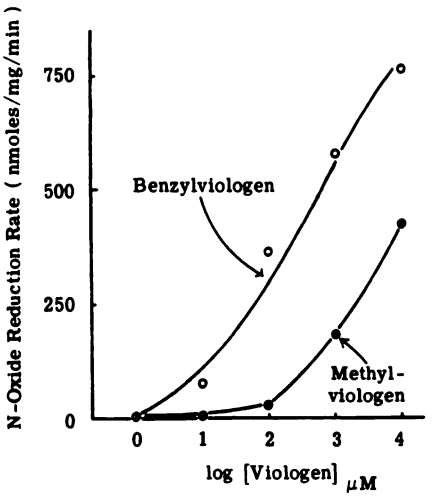

FIG. 6. *Effect of viologen concentration on xanthine oxidase-supported tiaramide N-oxide reduction by liver microsomes*

The incubation mixture was the same as in Fig. 5. The incubation time was 5 min.

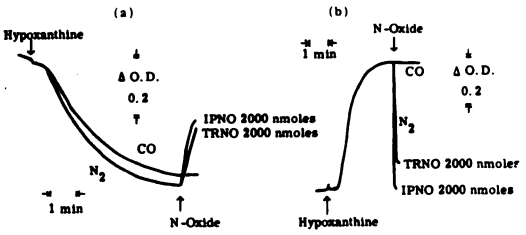

FIG. 7. *Xanthine oxidase-supported reductions of FMN and methyl viologen and their reoxidations by tertiary amine N-oxides by liver microsomes*

a. The presence of FMN. The incubation mixture (2.4 ml) consisted of 0.25 mg of protein, 125 nmoles of FMN, 1.2 units of xanthine oxidase, and 150 μmoles of phosphate buffer, pH 7.4. Hypoxanthine (10 μmoles), imipramine *N*-oxide (IPNO; 2 μmoles), and tiaramide *N*-oxide (TRNO; 2 μmoles) were added anaerobically at the indicated times. The change in absorbance at 450 nm was recorded under an atmosphere of nitrogen and carbon monoxide. The formation of imipramine and tiaramide was determined 1 min after addition of the *N*-oxides.

b. The presence of methyl viologen. The incubation mixture was the same as in Fig. 7a, except that methyl viologen (2.5 μmoles) was added instead of FMN. The formation of imipramine and tiaramide was determined 30 sec after addition of the *N*-oxides.

carbon monoxide atmosphere the reoxidation of reduced FMN was completely abolished, and the amounts of imipramine and tiaramide formed were 3% and 2%, respectively, of those obtained under nitrogen.

Xanthine oxidase-supported reduction of methyl viologen and its reoxidation by tertiary amine N-oxide in liver microsomes. Methyl viologen was rapidly reduced by xanthine oxidase on the addition of hypoxanthine under a nitrogen atmosphere, with a maximum rate of about 134 nmoles/min (Fig. 7b); however, only about 6% of the methyl viologen was in the reduced form in the steady state. The reduced methyl viologen was rapidly reoxidized by the addition of imipramine *N*-oxide and tiaramide *N*-oxide, with initial reoxidation rates of about 3.0 and 1.2 μmoles/mg of protein per minute, respectively. The rates of formation of imipramine and tiaramide under these conditions were 0.63 and 0.54 μmole/mg of protein per 30 sec, respectively. Under a carbon monoxide atmosphere the reoxidation of reduced methyl viologen was completely abolished, and the amounts of imipramine and tiaramide formed were 1% and 3%, respectively, of those obtained under nitrogen.

Effect of prior incubation on FMN- or methyl viologen-induced stimulation of xanthine oxidase-supported tiaramide N-oxide reduction. The results presented in Figs. 5–7 suggest that the stimulatory effect of FMN or methyl viologen on tiaramide *N*-oxide reduction was much higher after incubation with a reducing agent. Since the incubation mixture contained only 0.1-nmole of cytochrome P-450, the contribution of previously existing reduced cytochrome P-450 to the reduction of tiaramide *N*-oxide was negligible. Therefore the amount of reduced FMN or methyl viologen, or the ratio of the reduced to the oxidized forms during incubation, may be important for the rate of cytochrome P-450 and *N*-oxide reduction. Prior incubation markedly enhanced the stimulatory effects of FMN and methyl viologen (Fig. 8). Methyl viologen-supported tiaramide *N*-oxide reduction was proportional to the amount of xanthine oxidase, and the effect of prior incubation seemed to be independent of the amount of xanthine oxidase, whereas FMN-supported *N*-oxide reduction was very slow at low concentrations of xanthine oxidase and the reduction rate

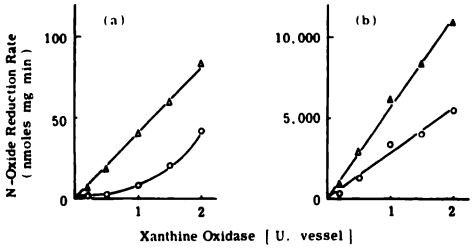

FIG. 8. *Effect of amount of xanthine oxidase on microsomal N-oxide reduction supported by FMN or methyl viologen*

a. FMN supported. The incubation mixture (2.5 ml) consisted of 1.25 mg of protein, 125 nmoles of FMN, 10 μmoles of hypoxanthine, 150 μmoles of phosphate buffer (pH 7.4), and various amounts of xanthine oxidase. Tiaramide *N*-oxide (5 μmoles) was added after a 10-min anaerobic incubation (△——△) or without prior incubation (○——○). The incubation was carried out anaerobically for 5 min.

b. Methyl viologen supported. The incubation mixture (2.5 ml) consisted of 0.125 mg of protein, 25 μmoles of methyl viologen, 10 μmoles of hypoxanthine, 150 μmoles of phosphate buffer (pH 7.4), and various amounts of xanthine oxidase. Tiaramide *N*-oxide (10 μmoles) was added after 5 min of anaerobic incubation (△——△) or without prior incubation (○——○). The incubation was carried out anaerobically for 2 min.

was proportional to the amount of xanthine oxidase after prior incubation. These results and the results shown in Figs. 1 and 2 indicate that a high ratio of reduced to oxidized FMN, rather than the absolute amount of reduced FMN, may be a determining factor in the reduction of cytochrome P-450 and *N*-oxide.

Effect of ratio of reduced to oxidized FMN on reduction of tertiary amine N-oxide. To obtain fully reduced FMN, the flavin was incubated with an equimolar amount of sodium dithionite for 1 min, and then with tiaramide *N*-oxide for 3 min. In contrast to the results shown in Fig. 1, the rate of tiaramide *N*-oxide reduction was parallel to the amount of added FMN (Fig. 9a). However, the reduction of tiaramide *N*-oxide was slower when the ratio of reduced to oxidized FMN was decreased (Fig. 9b). These results indicate that the rates of reduction of cytochrome P-450 and tiaramide *N*-oxide were determined by the ratio of reduced to oxidized FMN rather than by the amount of reduced FMN during the incubation period. Figure 10 shows the rate of formation of reduced FMN during an incubation started in the presence of a reducing agent (NADPH-cytochrome *c* reductase) and tiaramide *N*-oxide. In contrast to results in the absence of tiaramide *N*-oxide, very little FMN was in the reduced form in the presence of tiaramide *N*-oxide. However, the steady-state level of reduced FMN reached similar levels after the addition of tiaramide *N*-oxide.

NADPH-dependent reduction of FMN

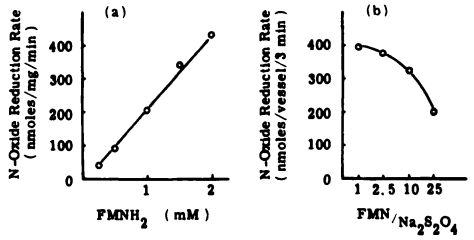

FIG. 9. *Effect of amount of reduced FMN on microsomal N-oxide reduction*

a. Effect of dithionate-reduced FMN concentration. The incubation mixture (2.5 ml) consisted of 2.5 mg of protein, 10 μmoles of tiaramide *N*-oxide, 150 μmoles of phosphate buffer (pH 7.4), and various amounts of FMN and sodium dithionite. The same amounts of FMN and sodium dithionite were incubated anaerobically for 1 min before the addition of tiaramide *N*-oxide. The incubation was carried out anaerobically for 3 min.

b. Effect of ratio of reduced to oxidized FMN. The incubation mixture (2.5 ml) consisted of 2.5 mg of protein, 10 μmoles of tiaramide *N*-oxide, 150 μmoles of phosphate buffer (pH 7.4), 0.5 μmole of sodium dithionite, and various amounts of FMN. FMN and sodium dithionite were incubated anaerobically for 1 min before the addition of tiaramide *N*-oxide. The incubation was carried out anaerobically for 3 min.

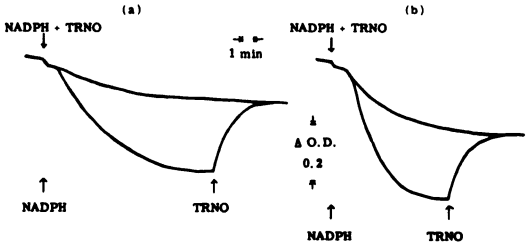

FIG. 10. *Reduction of FMN by liver microsomes during incubation with tiaramide N-oxide*

The incubation mixture consisted of 125 nmoles of FMN and other components described in MATERIALS AND METHODS. The amount of microsomal protein was 1.25 mg (a) or 2.5 mg (b). Tiaramide *N*-oxide (TRNO; 2 μmoles) was added simultaneously with NADPH or separately, as indicated.

and methyl viologen and their reoxidation by tiaramide N-oxide with a reconstituted cytochrome P-450 system. We previously reported (13) that a reconstituted system containing purified cytochrome P-450 and NADPH–cytochrome *c* reductase catalyzed the anaerobic reduction of tiaramide *N*-oxide by NADPH at a rate comparable to that obtained with intact liver microsomes. To confirm our hypothesis, we investigated the reduction of FMN and methyl viologen by purified NADPH–cyothrome *c* reductase and the reoxidation of reduced FMN and methyl viologen by cytochrome P-450-mediated tiaramide *N*-oxide reduction. Added FMN was slowly reduced by purified NADPH–cytochrome *c* reductase and rapidly reoxidized on the addition of tiaramide *N*-oxide (Fig. 11a). The maximal rate of reduction of FMN was 7.6 nmoles/min, and the initial reoxidation rate of reduced FMN was about 18 nmoles/min, or 54 nmoles/nmole of P-450 per minute. The rate of formation of tiaramide was 31 nmoles/nmole of P-450 per minute. On the other hand, added methyl viologen was rapidly reduced by purified NADPH–cytochrome *c* reductase and instantly reoxidized on the addition of tiaramide *N*-oxide (Fig. 11b). The maximum rate of reduction of methyl viologen was 40 nmoles/min, and the initial rate of reoxidation of reduced methyl viologen was about 58 nmoles/min, or 174 nmoles/nmole of P-450 per minute. The rate of formation of tiaramide was 148 nmoles/nmole of P-450 per minute, and this value was comparable to that obtained with intact rat and rabbit liver microsomes (13).

Xanthine oxidase-supported reduction of FMN and methyl viologen and their reoxidation by tiaramide N-oxide with purified cytochrome P-450. The xanthine oxidase-hypoxanthine system rapidly reduced FMN, which was reoxidized by the addition of tiaramide *N*-oxide (Fig. 12a). The maximum rate of reduction of FMN by this system was 33 nmoles/min, and the initial rate of reoxidation of reduced FMN was about 70 nmoles/min, or 420 nmoles/nmole of P-450 per minute. The rate of formation of tiaramide was 298 nmoles/nmole of P-450 per minute.

Similarly, the xanthine oxidase-hypoxanthine system rapidly reduced methyl viologen, which was reoxidized by the addition of tiaramide *N*-oxide (Fig. 12b). The maximum rate of reduction of methyl viologen by this system was 97 nmoles/min, and the initial rate of reoxidation of reduced methyl viologen was about 304 nmoles/min, or 1825 nmoles/nmole of P-450 per minute. The rate of formation of tiaramide was 1143 nmoles/nmole of P-450 per minute, and this value was comparable to that obtained with intact rat and rabbit liver microsomes (13).

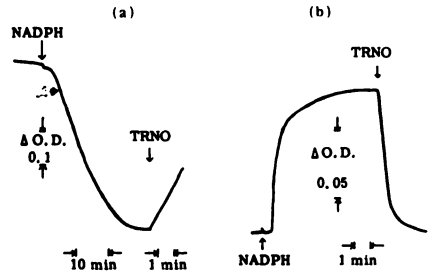

FIG. 11. *NADPH-dependent reduction of FMN and methyl viologen and their reoxidation by tiaramide N-oxide with a reconstituted cytochrome P-450 system*

The incubation mixture (2.4 ml) consisted of 0.2 unit of partially purified NADPH–cytochrome *c* reductase (11 units/mg), 0.33 nmole of cytochrome P-450 (17 nmoles/mg), 60 μmoles of phosphate buffer (pH 7.4), 0.001% Emulgen 913, and 125 nmoles of FMN (a) or 2.5 μmoles of methyl viologen (b). NADPH (2.5 μmoles) and tiaramide *N*-oxide (TRNO; 2 μmoles) were added at the indicated times.

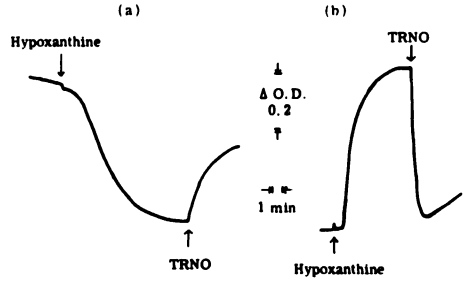

FIG. 12. *Xanthine oxidase-supported reduction of FMN and methyl viologen and their reoxidation by tiaramide N-oxide with purified cytochrome P-450*

The incubation mixture (2.4 ml) consisted of 0.163 nmole of purified cytochrome P-450 (17 nmoles/mg), 60 μmoles of phosphate buffer (pH 7.4), 0.001% Emulgen 913, 1.2 units of xanthine oxidase, and 125 nmoles of FMN (a) or 2.5 μmoles of methyl viologen (b). Hypoxanthine (10 μmoles) and tiaramide *N*-oxide (TRNO; 2 μmoles) were added at the indicated times.

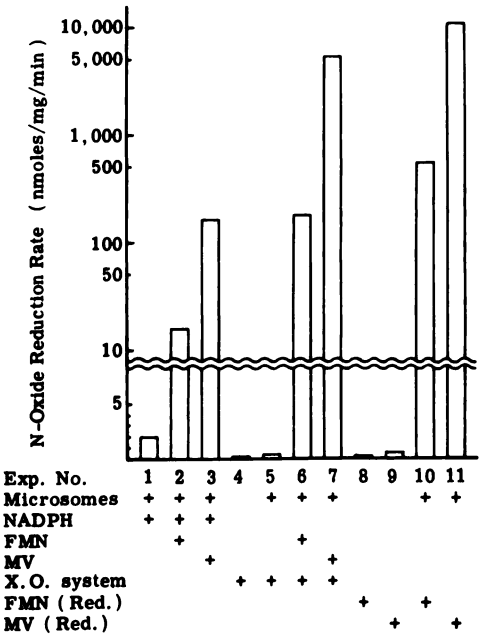

FIG. 13. *Effects of FMN and methyl viologen on NADPH- and xanthine oxidase-supported reduction of tiaramide N-oxide under optimal incubation conditions*

The incubation conditions were as follows: in experiment 1, microsomal protein (2.5 mg), NADPH (5 μmoles), tiaramide *N*-oxide (2.5 μmoles), incubation period, 5 min; in experiment 2, microsomal protein (2.5 mg), NADPH (5 μmoles), FMN (125 nmoles), tiaramide *N*-oxide (2.5 μmoles), incubation period, 5 min; in experiment 3, microsomal protein (1.25 mg), NADPH (5 μmoles), methyl viologen (MV; 25 μmoles), tiaramide *N*-oxide (10 μmoles), incubation period, 5 min; in experiment 4, xanthine oxidase (X.O; 2 units), hypoxanthine (10 μmoles), tiaramide *N*-oxide (2.5 μmoles), incubation period, 5 min; in experiment 5, microsomal protein (1.25 mg), xanthine oxidase (2 units), hypoxanthine (10 μmoles), tiaramide *N*-oxide (2.5 μmoles), incubation period, 5 min; in experiment 6, microsomal protein (1.25 mg), xanthine oxidase (2 units), hypoxanthine (10 μmoles), FMN (12.5 μmoles), tiaramide *N*-oxide (10 μmoles), incubation period, 5 min; in experiment 7, microsomal protein (0.5 mg), xanthine oxidase (2 units), hypoxanthine (10 μmoles), methyl viologen (25 μmoles), tiaramide *N*-oxide (10 μmoles), incubation period, 5 min; in experiment 8, reduced FMN (5 μmoles), tiaramide *N*-oxide (10 μmoles), incubation period, 1 min; in experiment 9, reduced methyl viologen (10 μmoles), tiaramide *N*-oxide (10 μmoles), incubation period, 1 min; in experiment 10, microsomal protein (0.5 mg), reduced FMN (5 μmoles), tiaramide *N*-oxide (10 μmoles), incubation period, 1 min; in experiment 11, microsomal protein (0.25 mg), reduced methyl viologen (10 μmoles), tiaramide *N*-oxide (10 μmoles), incubation period, 1 min. FMN and methyl viologen were reduced anaerobically by equimolar sodium dithionite 1 min before the incubations.

Comparative studies on effect of FMN and methyl viologen on NADPH- and xanthine oxidase-supported tiaramide N-oxide reduction under optimal incubation conditions. The effects of FMN and methyl viologen on NADPH- or xanthine oxidase-supported tiaramide *N*-oxide reduction by hepatic microsomes under optimal incubation conditions are summarized in Fig. 13. The xanthine oxidase system alone and with the addition of microsomes reduced almost no tiaramide *N*-oxide, and chemically reduced FMN and methyl viologen reduced very little tiaramide *N*-oxide in the absence of microsomes. Maximal activity was obtained with reduced methyl viologen plus microsomes. The estimated turnover number of cytochrome P-450 was about 12,000/min. The stimulatory effect of reduced FMN and methyl viologen was completely abolished under an atmosphere of carbon monoxide.

DISCUSSION

It has been reported that hepatic microsomal cytochrome P-450 catalyzes the reduction of tertiary amine *N*-oxides to their corresponding amines (1, 2). The reduction is NADPH-dependent, and stoichiometric studies indicated that 1 mole of NADPH is oxidized for each mole of the corresponding amine formed from the *N*-oxide (2). The reduction of tertiary amine *N*-oxide is strongly inhibited by carbon monoxide and greatly stimulated by flavins (1–4).

The present studies have established that microsomal NADPH-cytochrome *c* reductase reduces added riboflavin, FMN, and FAD; the reduced flavins reduce cytochrome P-450, which in turn reduces tertiary amine *N*-oxides to the corresponding amines. Methyl viologen and benzyl viologen possess even stronger reductive effects. This evidence has been confirmed by results obtained with purified NADPH-cytochrome *c* reductase and cytochrome P-450 and by spectrophotometric studies.

A hepatic microsomal suspension or NADPH–cytochrome *c* reductase rapidly reduced added FMN in the presence of NADPH (Figs. 3a and 11). The xanthine oxidase system also reduced FMN (Fig. 7a). NADPH–cytochrome *c* reductase, the xanthine oxidase system, and reduced FMN, however, could not reduce tiaramide *N*-oxide directly, and the stimulatory effect of FMN required the presence of microsomal cytochrome P-450 (Fig. 13).

In addition, the stimulatory effects of riboflavin, FMN, and FAD on NADPH-supported tiaramide *N*-oxide reduction markedly decreased at high concentrations of the flavins (Fig. 1). These results suggest that the rate of reduction of cytochrome P-450 by the reduced flavins might depend on the ratio of reduced to oxidized flavin rather than on the absolute amount of reduced flavin. Indeed, the xanthine oxidase system reduced FMN more rapidly than did the NADPH–cytochrome *c* reductase system (Figs. 3a and 7a), and the stimulatory effects of flavins on xanthine oxidase-supported tiaramide *N*-oxide reduction paralleled the concentration of flavins over a relatively wide range (Fig. 5).

Finally, it was demonstrated that *N*-oxide reduction increases in parallel with the amount of dithionite-reduced FMN (Fig. 9a) and decreases as the ratio of reduced to oxidized FMN is decreased (Fig. 9b). These results indicate the presence of competition between oxidized and reduced FMN for the reduction of cytochrome P-450.

Methyl viologen was rapidly reduced by microsomes and microsomal NADPH–cytochrome *c* reductase in the presence of NADPH (Figs. 4a and 11b). However, reduced methyl viologen reduced very little tiaramide *N*-oxide in the absence of cytochrome P-450 (Figs. 12b and 13). The xanthine oxidase system reduced tiaramide *N*-oxide more rapidly through the reduction of methyl viologen in the presence of cytochrome P-450 (Figs. 7b, 12b, and 13).

All these results clearly indicate that the rate of reduction of tiaramide *N*-oxide is dependent on the rate of cytochrome P-450 reduction. The maximum turnover number of cytochrome P-450 in rat liver microsomes is about 12,000/min. These results therefore indicate that the substrate-binding and metabolite-releasing steps of cytochrome P-450 during tiaramide *N*-oxide reduction are relatively fast. The oxidation-reduction potential of cytochrome P-450 is about 340–400 mV (14). Therefore cytochrome P-450 is not readily reduced by NADPH–cytochrome P-450 reductase, although reduced cytochrome P-450 can be reoxidized easily by molecular oxygen, tertiary amine *N*-oxides, and arene epoxides (2, 15).

The maximum turnover number of cytochrome P-450 in rat liver microsomes in the presence of substrates, oxygen, and NADPH has been shown to be about 10–15/min (16, 17). Therefore the maximum turnover number of cytochrome P-450 obtained in the present studies was stimulated about 1000-fold.

The rate-limiting step in NADPH-dependent drug oxidation reactions of hepatic microsomes has not been definitely determined. Some investigators have claimed that the rate-limiting step appears to be the introduction of the first electron into cytochrome P-450 by NADPH–cytochrome P-450 reductase, but other have proposed another step (16–20).

Kamataki *et al.* (21) suggested that in whole microsomes the reduction of cytochrome P-450 is the rate-limiting step, since the addition of NADPH–cytochrome *c* reductase to the reconstituted system to give a reductase to cytochrome P-450 ratio above the microsomal level increased the activity of benzphetamine *N*-demethylase. In addition, Miwa and Cho (22) found that the addition of solubilized NADPH–cytochrome *c* reductase increased the endogenous activity of *N,N*-dimethylamphetamine *N*-demethylase about 5-fold. These results support the view that the rate-limiting step appears to be the reduction of cytochrome P-450 by NADPH–cytochrome P-450 reductase.

The results reported here also clearly indicate that the rate of reduction of tertiary amine *N*-oxides is closely related to the rate of reduction of cytochrome P-450 and that the maximum turnover number of

cytochrome P-450 appears to be more than 10,000/min.

These results provide valuable information for further kinetic studies on the relationship between the microsomal electron transport system and the oxidation-reduction cycle of cytochrome P-450 during the oxidation of drug substrates. Further kinetic studies with a stopped-flow apparatus on intact microsomes and the purified cytochrome P-450 system will be required.

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REFERENCES

1. Sugiura, M., Iwasaki, K., Noguichi, H. & Kato, R. (1974) *Life Sci.*, **15**, 1433–1442.
2. Sugiura, M., Iwasaki, K. & Kato, R. (1976) *Mol. Pharmacol.* **12**, 322–334.
3. Kato, R., Iwasaki, K. & Noguichi, H. (1976) *Biochem. Biophys. Res. Commun.*, **72**, 267–274.
4. Fishman, V. & Goldenberg, H. (1962) *J. Pharm. Pharmacol.*, **14**, 187–190.
5. Imai, Y. & Sato, R. (1974) *Biochem. Biophys. Res. Commun.*, **60**, 8–14.
6. Imai, Y. (1976) *J. Biochem. (Tokyo)*, **80**, 267–276.
7. Gillette, J. R., Kamm, J. J. & Sasame, H. A. (1968) *Mol. Pharmacol.*, **4**, 541–548.
8. Omura, T. & Sato, R. (1964) *J. Biol. Chem.*, **239**, 2379–2385.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265–275.
10. Kamm, J. J. & Gillette, J. R. (1963) *Life Sci.*, **4**, 254–260.
11. Bus, J. S., Aust, S. D. & Gibson, J. E. (1974) *Biochem. Biophys. Res. Commun.*, **58**, 749–755.
12. Ichikawa, Y. & Yamano, T. (1972) *J. Biochem. (Tokyo)*, **71**, 1053–1063.
13. Iwasaki, K., Noguchi, H., Kato, R., Imai, Y. & Sato, R. (1977) *Biochem. Biophys. Res. Commun.*, **77**, 1143–1149.
14. Waterman, M. R. & Mason, H. S. (1972) *Arch. Biochem. Biophys.*, **150**, 57–63.
15. Kato, R., Iwasaki, K., Shiraga, T. & Noguchi, H. (1976) *Biochem. Biophys. Res. Commun.*, **70**, 681–687.
16. Gigon, P. L., Gram, T. E. & Gillette, J. R. (1969) *Mol. Pharmacol.*, **5**, 109–122.
17. Schenkman, J. B. (1972) *Mol. Pharmacol.*, **8**, 178–188.
18. Masters, B. S. S., Nelson, E. B., Schacter, B. A., Baron, J. & Isaacson, E. L. (1973) *Drug Metab. Disp.*, **1**, 121–128.
19. Guengerich, F. P., Ballaou, D. P. & Coon, M. J. (1976) *Biochem. Biophys. Res. Commun.*, **70**, 951–956.
20. Matsubara, T., Baron, J., Peterson, L. L. & Peterson, J. A. (1976) *Arch. Biochem. Biophys.*, **172**, 463–469.
21. Kamataki, T., Lee Lin, M. C. M., Belcher, D. H. & Neal, R. A. (1976) *Drug Metab. Disp.*, **4**, 180–189.
22. Miwa, G. T. & Cho, A. K. (1976) *Life Sci.*, **18**, 983–988.