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Inhibition of Azoreductase by Oxygen

The Role of the Azo Anion Free Radical Metabolite in the Reduction of Oxygen to Superoxide

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SUMMARY

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Microsomal azo reduction of the diazonaphthol dye sulfonazo III is strongly inhibited by oxygen. The sulfonazo III anion radical metabolite, which is apparently the first intermediate of azoreductase activity, appears to react with oxygen in aerobic microsomal incubations. Even though the sulfonazo III is unchanged in aerobic NADPH-supplemented microsomal incubations, oxygen consumption increases 10-fold to 122 nmoles/min/mg of protein. This stimulated oxygen consumption is partially reversed by either superoxide dismutase or catalase. Concomitant with the increase in oxygen uptake is a 9-fold increase in the rate of superoxide formation. The oxidation of NADPH is also greatly increased by sulfonazo III, but is not influenced by superoxide dismutase or catalase. These results suggest that futile formation of the sulfonazo III anion free radical mediates oxygen reduction to $\dot{O}_2^-$ and NADPH oxidation, and that NADPH is not being oxidized by a superoxide-NADPH chain reaction.

INTRODUCTION

Recent studies from our laboratory have shown that a diazonaphthol dye, sulfonazo III, is rapidly reduced by hepatic microsomes under nitrogen or carbon monoxide (1). Furthermore, in these anaerobic incubations we have observed an ESR spectrum that we have identified on the basis of its *g* value as the azo anion free radical metabolite of the dye. These studies suggested that the dye is reduced to the free radical metabolite by the transfer of a single electron from NADPH-cytochrome *c* reductase. The free radical can then disproportionate to give the corresponding hydrazine (2) or react with oxygen to regenerate the parent compound (3, 4).

The present investigations suggest that sulfonazo III is also reduced to an azo anion free radical in aerobic microsomal incubations. The reaction of this free radical with oxygen appears to mediate the reduction of oxygen to the superoxide anion free radical, $\dot{O}_2^-$ (pathway I, Fig. 1). This air oxidation of the radical metabolite regenerates the parent azo compound and appears to be responsible for the oxygen inhibition of microsomal azo reduction of sulfonazo III, and possibly other oxygen-sensitive azoreductases (7-9).

Hernandez, Gillette, and Mazel (7), in their studies of the azo reduction of neoprotosil, postulated a similar mechanism for

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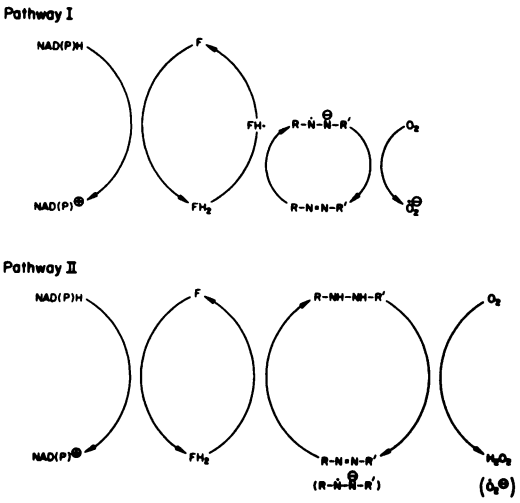

FIG. 1. *Two proposed mechanisms of azo compound-mediated production of superoxide anion radical and/or hydrogen peroxide and oxygen consumption*

Pathway II assumes that the corresponding hydrazine is the first intermediate of azo reduction, whereas pathway I assumes that the azo anion free radical precedes the formation of the hydrazine intermediate. The exact scheme of electron donation via NADPH-cytochrome *c* reductase does not alter our conclusions (5, 6).

the inhibition of the reduction of this drug. They proposed that the first intermediate in the reduction was a 2-electron reduction product, the corresponding hydrazine derivative. They further suggested that the oxygen inhibition of the reduction resulted from the oxidation of this intermediate (pathway II, Fig. 1). In both schemes the futile metabolism of the dye would lead to the stimulation of oxygen reduction to superoxide and/or hydrogen peroxide and the oxidation of NADPH.

A third mechanism for the inhibition by oxygen of the reduction of sulfonazo III could be that the reduction is catalyzed by an air-oxidizable enzyme or cofactor. Air oxidation of reduced cytochrome P-450 (10) and of a flavin cofactor (9) has been reported to inhibit azo reduction. In such cases the azo compound is not expected to increase the uptake of oxygen, the formation of superoxide or hydrogen peroxide, or the oxidation of NADPH.

In the current study we have examined the effect of this dye on oxygen uptake, NADPH oxidation, and the formation of superoxide by microsomes. These data, in addition to our previous observation of an apparent reaction between the sulfonazo III anion radical metabolite and oxygen (1), strongly suggest that the inhibition of this microsomal azoreductase by oxygen is the result of the air oxidation of the azo anion radical metabolite. Our findings further suggest that the azo anion free radical is an obligate intermediate in the reduction of sulfonazo III.

METHODS

Hepatic microsomes were prepared from 160–180-g, fed male C-D rats (Charles River). The livers were homogenized in 3 volumes of 150 mM KCl–50 mM Tris, pH 7.4. The homogenates were centrifuged at 9000 × *g* for 15 min, the pellet was discarded, and the supernatant was centrifuged at 165,000 × *g* for 40 min. The microsomal pellets were resuspended in KCl-Tris and resedimented at 165,000 × *g*. The microsomal protein was determined by the method of Sutherland *et al.* (11).

ESR spectra of the free radicals were obtained in anaerobic incubations at 25° with a Varian E-4 spectrometer (12). Oxygen uptake was determined with a Clark electrode (YSI-5331, Yellow Springs Instrument Company) in a water-jacketed glass vessel maintained at 37° and filled with 150 mM KCl–50 mM Tris–5 mM $MgCl_2$, pH 7.4. Microsomes were added through an injection port to give a concentration of 1 mg/ml. The reaction was initiated by the addition of NADPH to 0.13 mM, and the rate was taken as the initial slope. When substrates, superoxide dismutase (Miles Laboratories), or catalase (Sigma Chemical Company; thymol-free) were included, they were added before the microsomes.

The rate of oxidation of NADPH was determined essentially as previously described (13). In this assay 150 mM KCl–50 mM Tris–5 mM $MgCl_2$ buffer (pH 7.4) was warmed to 37° in a 1-cm cuvette and then placed in an Aminco DW2 spectrophotometer in the dual-wavelength mode, with the cell block maintained at 37°. A suspension of microsomal protein was then added to give a concentration of 1 mg/ml, followed

by sulfonazo III, superoxide dismutase, or catalase, as indicated. After 1 min, 10 μl of NADPH (39 mM) were added to give a final concentration of 0.13 mM, and the change in absorbance between 340 and 390 nm was followed for up to 2 min. The rate of oxidation of NADPH was determined from the initial slope, using an extinction coefficient of 6.2 mM^{-1} cm^{-1}.

The adrenochrome assay for $\dot{O}_2^-$ was a modification of the method of Misra and Fridovich (14). This assay was performed at 37° in an Aminco DW2 spectrophotometer in the split-beam mode. A solution of epinephrine (30 μl, 0.02 M in 0.02 N HCl) and the microsomal preparation were added to both cuvettes. One minute later NADPH (0.39 mM) was added to the sample side only, and the absorbance at 480 nm was followed. The rate of adrenochrome formation was determined using an extinction coefficient of 4.02 mM^{-1} cm^{-1} (15). The addition of the acidic solution of epinephrine did not affect the pH of the final solution. The formation of adrenochrome by NADPH-supplemented microsomal incubations containing epinephrine is sigmoidal, and is apparently autocatalytic under these conditions. The maximal rate occurs after a time lag of as much as 5 min. In the presence of 50 μM sulfonazo III the initial rate of formation of adrenochrome is also the maximal rate. We have reported initial rates determined within the first 30 sec of the reaction.

RESULTS

Anaerobic microsomal incubations containing sulfonazo III and NADPH have ESR spectra of the sulfonazo III anion free radical (Fig. 2). Since the amplitude of these spectra correlates with the rate of dye disappearance in microsomal incubations, we have proposed that the free radical is an obligate intermediate of microsomal reduction (1). In aerobic microsomal incubations the free radical could not be observed and the rate of dye disappearance was markedly decreased (Table 1).

If the sulfonazo anion free radical reacts with oxygen to regenerate the parent compound and form superoxide, the sulfonazo III might stimulate oxygen uptake without itself being consumed. Indeed, the NADPH-supported uptake of oxygen by hepatic microsomal preparations was increased 10-fold by 50 μM sulfonazo III (Table 2). Under these conditions the rate of azo dye disappearance is only 1.8 nmoles/min/mg (Table 1), which is only 1.5% of the rate of oxygen consumption (122.6 nmoles of O_2 per minute per milligram) (Table 2). This implies that the consumption of oxygen is indeed catalytic, as is consistent with the pathways of Fig. 1.

In order to test for the predicted forma-

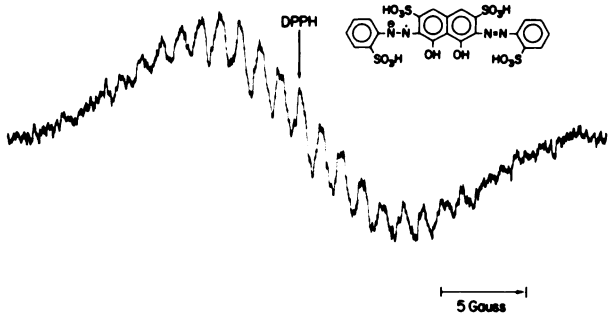

FIG. 2. *ESR spectrum of sulfonazo III anion free radical metabolite formed in the first microsomal incubation described in Table 1*

The *g* value of 2,2-diphenyl-1-picrylhydrazyl (DPPH) is indicated by the arrow. The nominal microwave power was 0.5 mW, and the modulation amplitude was 0.8 G. The 17 partially resolved lines of the hyperfine pattern indicate that the unpaired electron is delocalized onto at least one of the aromatic rings and probably onto both azo groups. If the unpaired electron is distributed throughout the free radical, as is likely, the theoretical hyperfine pattern would consist of 6075 lines. The assignment of the unpaired electron to one of the azo groups is arbitrary and made only for the sake of convenience.

TABLE 1

Effect of atmospheric conditions on disappearance of sulfonazo III and on steady-state ESR signal of sulfonazo III anion free radical metabolite in rat hepatic microsomal incubations

Atmosphere	Dye disappearance[a]	Relative amplitude[b]
	%	%
N_2	100.0 ± 0.8	100.0 ± 0.1
Air	3.1 ± 0.2	7.3 ± 5.2
CO	88.2 ± 0.7	96.0 ± 0.9
N_2 (microsomes heated at 57° for 15 min)	3.1 ± 0.3	1.0 ± 0.2

[a] The value for dye disappearance under an N_2 atmosphere was 59.5 ± 0.8 nmoles/min/mg of protein (average ± standard error of triplicate incubations, with ϵ = 19.1 mM^{-1} cm^{-1} at 573 nm). Reactions were performed at 37°; 3-ml incubations contained 50 μM sulfonazo III, 100 μg of protein per milliliter, 0.39 mM NADPH, and 150 mM KCl–50 mM Tris buffer, pH 7.4.

[b] Instrument settings of 5.0-mW microwave power and 20-G modulation amplitude, which maximized the signal-to-noise ratio, were used to monitor the steady-state ESR signal. Glucose 6-phosphate dehydrogenase (0.67 U/ml) was added to a 37° incubation containing 1.0 mg of protein per milliliter, 5 mM sulfonazo III, 0.87 mM NADP, and 10.9 mM glucose 6-phosphate. A 1.5-min interval was required for full activity, apparently because of inhibition by NADP. Even with an NADPH-generating system the steady-state signal decreased, and after 4 min it was 92% of the maximum value. The values reported are means of duplicates ± one-half the range. Sulfonazo III (5 mM) in 150 mM KCl–50 mM Tris–5 mM $MgCl_2$ buffer, pH 7.4, had a signal of relative amplitude 1.9 from a trace of a stable free radical impurity. This signal was not present in buffer and was subtracted from all the relative amplitudes above.

tion of superoxide, superoxide dismutase was added to these incubations and was found to decrease the sulfonazo III stimulation[1] of oxygen uptake by 10%. The relatively small effect of superoxide dismutase on oxygen uptake is most likely due to the spontaneous disproportionation of $\dot{O}_2^-$.

$$2\,\dot{O}_2^- + 2\,H^+ \xrightarrow{k_d} O_2 + H_2O_2$$

The k_d for this reaction is 2×10^5 M^{-1} sec^{-1} at pH 7.4 (16). For such a rapid spontaneous reaction it may seem surprising that $\dot{O}_2^-$ could accumulate to an extent that would affect the oxygen uptake as measured by the Clark electrode. Within the 15 sec required to make a measurement of oxygen uptake with our Clark electrode, the $\dot{O}_2^-$ had reached a relatively low steady-state concentration. Since only hydrogen peroxide can accumulate, oxygen consumption of 122 nmoles/min/mg implies a rate of $\dot{O}_2^-$ formation of at least 244 nmoles/min/mg.

$$2\,O_2 \xrightarrow{k} 2\,\dot{O}_2^- \xrightarrow{k_d} H_2O_2 + O_2$$

From the steady-state condition, where $(\dot{O}_2^-)_{ss}$ is the steady-state concentration of $\dot{O}_2^-$ and k is the rate of superoxide formation,

$$\frac{d(\dot{O}_2^-)}{dt} = 0 = k - k_d(\dot{O}_2^-)_{ss}^2$$

the equation for $(\dot{O}_2^-)_{ss}$ can be derived.

$$(\dot{O}_2^-)_{ss} = \sqrt{k/k_d}$$

Substitution of 244 nmoles/min/mg as the rate of $\dot{O}_2^-$ formation and 2×10^5 M^{-1} sec^{-1} as the k_d into the equation for the steady-state superoxide anion concentration gives $(\dot{O}_2^-)_{ss}$ = 4.6 μM (17). The addition of superoxide dismutase in quantities sufficient to lower $(\dot{O}_2^-)_{ss}$ to zero would recover half the lost oxygen. Therefore the difference in oxygen uptake with and without superoxide dismutase is expected to be 2.3 nmoles/ml

TABLE 2

Effect of superoxide dismutase and catalase on sulfonazo III stimulation of oxygen uptake by rat hepatic microsomal incubations

Values of oxygen uptake are averages ± standard errors of triplicate incubations. By two-way analysis of variance the catalase effect was significant to $p < 0.001$, the superoxide dismutase effect to $p < 0.005$, and the interaction between superoxide dismutase and catalase to $p < 0.05$.

Sulfonazo III (50 μM)	Superoxide dismutase (40 μg/ml)	Catalase (67 μg/ml)	O_2 uptake
			nmoles/min/mg protein
–	–	–	12.2 ± 0.2
+	–	–	122.6 ± 1.2
+	+	–	111.2 ± 0.6
+	–	+	83.1 ± 1.9
+	+	+	79.3 ± 1.9

[1] Stimulated oxygen uptake is the oxygen uptake in the presence of sulfonazo III minus that in its absence.

in the first 15 sec, which is equivalent to an initial rate of 9.2 nmoles/min/mg, in good agreement with Table 2.

As expected, the addition of catalase alone also significantly decreased the stimulation of oxygen uptake (36%) by increasing the rate of disproportionation of the hydrogen peroxide formed spontaneously from O_2^-.

$$2\,H_2O_2 \xrightarrow{\text{catalase}} O_2 + 2\,H_2O$$

Alternative explanations for the decreases in oxygen uptake caused by superoxide dismutase are possible. If the superoxide formed by the air oxidation of the azo anion free radical can catalyze the oxidation of NADPH by oxygen (Fig. 3), as is the case for lactate dehydrogenase-bound NADH (18), the superoxide dismutase would inhibit this additional oxygen uptake by inhibiting NADPH oxidation. Alternatively, superoxide dismutase may directly inhibit electron transport by the azoreductase. Both these possibilities imply that superoxide dismutase will inhibit the sulfonazo III stimulation of NADPH oxidation. Yet NADPH oxidation, in contrast to oxygen uptake, is insensitive to superoxide dismutase (Table 3). If a superoxide-NADPH chain reaction occurs at all in rat hepatic microsomal incubations (Fig. 3), the rate of NADPH oxidation to NADP$^\cdot$ is insignificant in comparison with the NADPH oxidation resulting from the futile azo reduction. In addition, neither catalase nor superoxide dismutase plus catalase has a significant effect upon the sulfonazo III-stimulated NADPH oxidation. These results suggest that NADPH is not significantly oxidized by either superoxide or hydrogen peroxide or by any subsequently derived species, such as the hydroxyl radical.

A key prediction of our mechanism of oxygen inhibition of microsomal azoreductase is that of superoxide anion free radical formation. The initial rate of epinephrine oxidation to adrenochrome is markedly stimulated by the addition of sulfonazo III to the incubation (Table 4). Furthermore, this stimulation is eliminated by the addition of superoxide dismutase. The air oxidation of the hydrazine intermediate could be responsible for the observed superoxide formation. Trace quantities of the hydra-

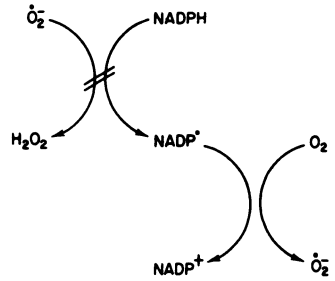

FIG. 3. *Absence of a microsomal superoxide-NADPH chain reaction*

In rat hepatic microsomal incubations, neither superoxide nor any subsequently derived species (H_2O_2, $^\cdot OH$, etc.) appears to promote the rate of NADPH oxidation via a free radical chain reaction as occurs in solutions of lactate dehydrogenase (18). In our view, the stimulation of NADPH oxidation by sulfonazo III in microsomal incubations is predominantly the result of the futile reduction of sulfonazo III to its anion free radical.

TABLE 3

Effect of superoxide dismutase and catalase on sulfonazo III stimulation of NADPH oxidation by rat hepatic microsomal incubations

Values are averages ± standard errors of triplicate incubations. None of the sulfonazo III values is significantly different by a two-way analysis of variance.

Sulfonazo III (50 μM)	Superoxide dismutase (40 μg/ml)	Catalase (67 μg/ml)	NADPH oxidation
			nmoles/min/mg protein
−	−	−	9.59 ± 0.41
+	−	−	74.05 ± 1.16
+	+	−	71.14 ± 1.34
+	−	+	71.71 ± 1.98
+	+	+	69.34 ± 1.67

TABLE 4

Effect of superoxide dismutase on initial rate of adrenochrome formation in rat hepatic microsomal incubations

Values are averages ± standard errors of triplicate incubations.

Superoxide dismutase	Adrenochrome formation	
	Basal	Sulfonazo III (50 μM)
μg/ml	*nmoles/min/mg protein*	
0	2.6 ± 0.2	24.1 ± 0.9
1	0	4.7 ± 0.6
5	0	0.2 ± 0.2

zine intermediate, formed in the presence of oxygen, could be postulated to catalyze oxygen uptake and superoxide generation (pathway II, Fig. 1), as has been observed for the air oxidation of phenylhydrazine (19, 20). Pathway II is apparently unimportant, because although sulfonazo III reduction by NADPH presumptively proceeds as a 2-electron reduction resulting in the formation of the hydrazine intermediate, this reaction is oxygen-insensitive. Aerobic microsomal incubations reduce sulfonazo III at the same rate as the nonenzymatic reaction, but the incubations containing NADPH alone do not consume significant quantities of oxygen even at 10 mM NADPH (Fig. 4). Microsomal incubations containing 0.13 mM NADPH consume oxygen at the rate of 122.6 nmoles of O_2 per minute per milligram (Table 2), whereas in the absence of microsomes there is no detectable oxygen uptake (Fig. 4).

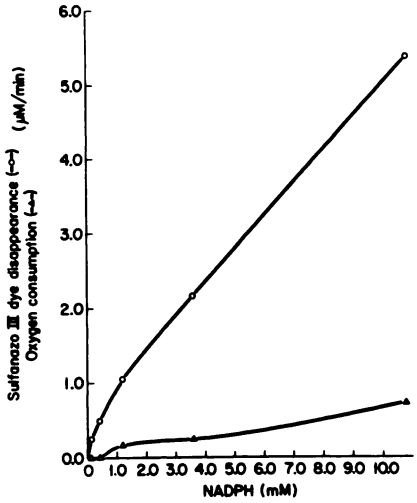

FIG. 4. *Oxygen consumption during the oxygen-insensitive nonenzymatic azo reduction*

Under aerobic conditions, at concentrations of NADPH greater than 0.1 mM, sulfonazo III (50 μM) is reduced nonenzymatically in 150 mM KCl–50 mM Tris–5 mM $MgCl_2$, pH 7.4. This reduction is oxygen-insensitive (1) and, in comparison with microsomal azo reduction (Table 2), does not cause significant oxygen uptake.

DISCUSSION

Azo dyes and drugs are known to be reductively cleaved by a variety of biological systems (21–23). In addition to the amine cleavage products and the hydrazine intermediate, we have detected by ESR an azo anion radical metabolite of sulfonazo III in microsomal incubations under nitrogen or carbon monoxide (1). The CO-insensitive microsomal azoreductase has been shown to be primarily NADPH–cytochrome *c* reductase (7, 24). These data suggest that this azo reduction, as in the reduction of cytochrome *c* or P-450 (25, 26), proceeds by a 1-electron transfer from the microsomal flavoprotein NADPH–cytochrome *c* reductase to form the azo anion radical metabolite (Figs. 1 and 2). Sulfonazo III, like cytochrome *c*, appears to be an alternative electron acceptor to cytochrome P-450. In aerobic microsomal incubations, oxygen consumption, superoxide formation, and NADPH oxidation are increased an order of magnitude over basal levels by 50 μM sulfonazo III, presumably by the sequence of reactions depicted in pathway I of Fig. 1.

In aerobic microsomal incubations, the sulfonazo anion free radical reacts with oxygen, resulting in its immediate air oxidation, as one would expect from chemical studies (3, 4). The air oxidation of the azo free radical metabolite results in the catalytic reduction of molecular oxygen to superoxide and may be responsible for the oxygen inhibition of many azoreductases (7–9). Earlier, methyl orange had been reported to increase oxygen consumption by various rat liver preparations without undergoing a net change (27). These observations were interpreted by assuming that the dye acts as a hydrogen carrier to oxygen (i.e., 2-electron transfer) via reversible reduction to the hydrazine intermediate.

In anaerobic aqueous media the azobenzene anion free radical is known to disproportionate to form hydrazobenzene (2). Although reduction of azobenzene *in vivo* (28) and *in vitro* (8) also results in the formation of hydrazobenzene, the anion radical may not be an obligate intermediate in these cases. Not all azoreductases are oxygen-sensitive; in fact, several completely oxygen-insensitive azoreductases have been reported (29–32). In particular, the soluble fraction of rat liver contains an oxygen-in-

sensitive azoreductase, which is inhibited by dicoumarol and thought to be DT diaphorase (EC 1.6.99.2) (29, 30). In 9000 × *g* rat hepatic supernatant the relative importance of the oxygen-sensitive and -insensitive mechanisms depends on the substrate (8). Even microsomal azoreductase can be nearly oxygen-insensitive, as in the case of butter yellow azo reduction (30, 32). Previous investigations of the oxygen reactivity of the azo anion free radical make it unlikely that oxygen-insensitive azo reductases form the azo anion free radical intermediate (3, 4). These azoreductases presumably reduce azo compounds by a 2-electron transfer to form the hydrazine intermediate directly on the surface of the enzyme.

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