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Biochemical Pharmacology, Vol. 34, No. 16, pp. 3005–3008, 1985.
Printed in Great Britain.

Microsomal reduction of bisulfite (aqueous sulfur dioxide)—Sulfur dioxide anion free radical formation by cytochrome P-450

(*Received* 17 *October* 1984; *accepted* 1 *February* 1985)

In aqueous medium near pH 7, SO_2 exists primarily as sulfite ($SO_3^=$) and bisulfite (HSO_3^-) and, to a minor extent, as hydrated SO_2 [1, 2]. The nomenclature (bi)sulfite is used when it is not known which of these species, all of which have a sulfur atom with a formal oxidation state of plus four, is involved in a reaction.

The metabolism of (bi)sulfite has centered upon its oxidation to sulfate. Sulfite oxidase, a mitochondrial enzyme, is thought to be primarily responsible for the oxidation of (bi)sulfite *in vivo* [2, 3]. Sulfite oxidase is considered to be a two-electron acceptor of electrons from (bi)sulfite.

$$SO_3^= \rightarrow SO_3 + 2e^- \quad (1)$$

$$SO_3 + H_2O \rightarrow SO_4^= + 2H^+ \quad (2)$$

These reactions are thought to detoxify sulfite without forming free radical intermediates [4].

The one-electron oxidation of (bi)sulfite

$$SO_3^= \rightarrow \dot{S}O_3^- + e^- \quad (3)$$

is catalyzed by peroxidases. Either horseradish peroxidase [5, 6] or the prostaglandin H synthase (hydroperoxidase) in ram seminal vesicles or guinea pig lung microsomes [7] catalyzes the formation of the sulfur trioxide anion free radical ($\dot{S}O_3^-$) as demonstrated with ESR investigations. Autoxidation of (bi)sulfite is catalyzed by transition metals, but while this reaction is thought to form SO_3^-, it is not thought to be important *in vivo* [2, 4].

Although the biological oxidation of (bi)sulfite is certainly more common than (bi)sulfite reduction [1–3, 8], under anaerobic conditions (bi)sulfite is reduced to dithionite by either reduced flavodoxins or a mixture of paraquat, H_2, and hydrogenase [9]. In the latter case, the paraquat cation free radical reduces (bi)sulfite to form the sulfur dioxide anion free radical, which is in equilibrium with dithionite [10].

$$2\dot{S}O_2^- \rightleftarrows S_2O_4^= \quad (4)$$

Rat liver microsomes contain cytochrome P-450 and its flavin-containing reductase, NADPH-cytochrome P-450 reductase. The cytochrome P-450-dependent monooxygenase system is known to donate one electron to a few xenobiotics and, thereby, to form free radicals [11]. With this fact in mind, electron spin resonance was used to search for the sulfur dioxide anion free radical [the one-electron reduction product of (bi)sulfite] in rat hepatic microsomal incubations.

Materials and methods

Hepatic microsomes, mitochondria, and cytosol were prepared from fed, untreated Sprague–Dawley rats, as described [12], and kept on ice until used. ESR spectra were recorded with a Varian E-109 spectrometer equipped with an E-238 TM_{110} cavity. The g-value of the sulfur dioxide anion radical was determined relative to the g-value (2.0037) of 2,2-diphenyl-1-picrylhydrazyl (DPPH) dispersed in KCl. A capillary tube containing this secondary g-value standard was attached to an aqueous flat cell containing the hepatic protein incubation, and the unknown g-value was calculated as described in Ref. 13.

All experiments were performed at room temperature in either 0.1 M phosphate, Tris–HCl, or borate buffers (pH = 7.5) containing 1.0 mM diethylenetriaminepentaacetic acid (DETAPAC), which was necessary to prevent the autoxidation of (bi)sulfite during sample preparation [6]. An incubation contained ~1 mg/ml microsomal, mitochondrial, or cytosolic protein, 1.0 mM or 10 mM Na_2SO_3, 5.5 mM glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*, Sigma type XXIII), and 0.37 mM $NADP^+$ or NAD^+ in a total volume of 3 ml. The reaction was initiated by the addition of the pyridine nucleotide (10 μl of a 117 mM solution) under a nitrogen atmosphere. The flat cell was purged with nitrogen gas and then filled from the bottom using pressurized nitrogen gas to force the incubation into the flat cell, which was then sealed [14].

Results

After 14 min, microsomal incubations containing sodium sulfite and an NADPH-generating system developed a weak, broad ESR spectrum. This signal continued to increase linearly for hours (Fig. 1). The spectrum in Fig. 2A was obtained after 2 hr, and was completely dependent upon the presence of active microsomal protein (Fig. 2B), the NADPH-generating system (Fig. 2C), and sulfite (Fig. 2D). The sulfur dioxide radical anion has been reported to form nonenzymatically from (bi)sulfite (under unspecified conditions) in the presence of NADPH, NADH or ascorbate [15], but under our conditions absolutely no signal was detected in the absence of microsomal protein. Heat-

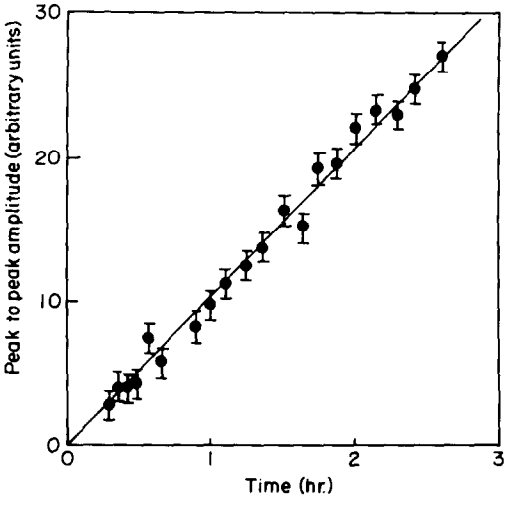

Fig. 1. Time course of the peak-to-peak amplitude of the sulfur dioxide anion radical under a nitrogen atmosphere at room temperature in 0.1 M borate buffer (pH 7.5) containing 1 mg/ml rat hepatic microsomal protein, 10 mM Na_2SO_3, and the NADPH-generating system described in Materials and Methods. The bars represent the range of duplicates. Instrumental conditions were: scan time, 8 min; modulation amplitude, 0.67 G; time constant, 1.0 sec; and microwave power, 20 mW.

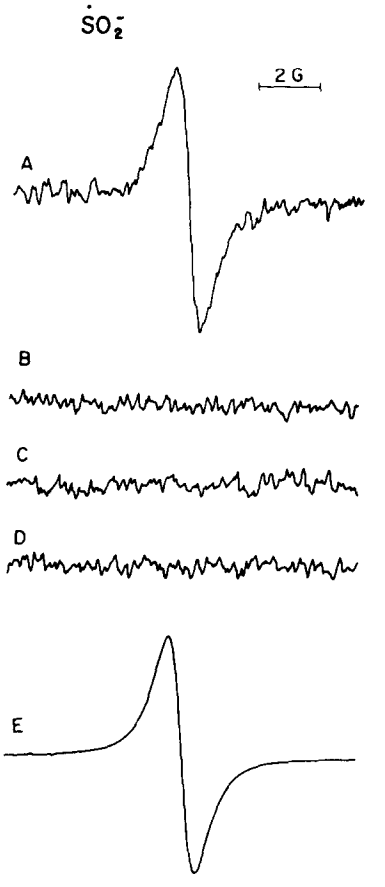

Fig. 2. Electron spin resonance spectra of the sulfur dioxide anion free radical. Key: (A) incubation containing 1 mg/ml microsomal protein, sodium sulfite (10 mM) in 0.1 M borate buffer (pH 7.5) and the NADPH-generating system described in Materials and Methods; (B) identical to A, but microsomes heated at 56° for 30 min; (C) identical to A, but NADPH-generating system deleted; (D) identical to A, but sodium sulfite deleted; and (E) borate buffer containing 1.4 mM sodium dithionite without microsomal protein. Instrumental conditions were: scan time, 8 min; modulation amplitude, 0.67 G; time constant, 1 sec; and microwave power, 20 mW. Receiver gain was 1.6×10^5 in A–D and 2.5×10^3 in E.

denaturing the protein at 56° for 30 min (Fig. 2B) or stirring the incubation in air also resulted in no detectable signal. The ESR spectrum was characterized by a g-value of 2.0057 ± 0.0001 and a peak-to-peak line width of 0.95 G. These parameters were identical to those of the spectrum obtained by dissolving a few milligrams of sodium dithionite in buffer (Fig. 2E). Sodium dithionite has an unusually long sulfur—sulfur bond, which led to the suggestion that homolytic bond cleavage would form $\dot{SO}_2^-$ [16]. The ESR spectrum of $\dot{SO}_2^-$ in anaerobic aqueous solutions of sodium dithionite is well known [17].

The common isotopes of sulfur and oxygen do not have nuclear spin; therefore, only the rare isotopes ^{33}S and ^{17}O yield the pattern of hyperfine lines characteristic of most ESR spectra. These weak satellite lines were below the sensitivity of the spectrometer and could not be detected in these incubations. However, the g-value of $\dot{SO}_2^-$ [(2.0058) [18]] is unique for sulfur-oxygen free radicals, with the g-values of $\dot{SO}_3^-$ (2.00306 [19]) and $\dot{SO}_4^-$ (2.0125 [20]) being distinctly different. We did not detect the NADPH-cytochrome P-450 reductase semiquinone under the conditions used in Fig. 2. Although this free radical is probably present, 53 mg/ml of phenobarbital-induced microsomes was required to obtain a signal [21]. In any case, the NADPH-cytochrome P-450 reductase semiquinone spectrum is characterized by a line width of 20 G [21], and is therefore clearly distinct from that of the sulfur dioxide anion free radical.

As shown in Fig. 1, the $\dot{SO}_2^-$ signal continued to increase over a period of hours. Microsomes kept under nitrogen at room temperature and in the presence of an NADPH-generating system were shown to have cytochrome P-450 for at least 6 hr (data not shown) using difference spectroscopy [22]. The $\dot{SO}_2^-$ signal that we observed is a cumulative signal, i.e., the system reduces $SO_3^=$ to $\dot{SO}_2^-$ as long as the enzyme is active. Since the $\dot{SO}_2^-$ radicals establish an equilibrium with dithionite, the ESR signal is detectable until the system is exposed to oxygen.

The equilibrium constant for the equilibrium between $\dot{SO}_2^-$ and dithionite (equation 4) has been determined with ESR as 1.4×10^{-9} M [23] and 5.1×10^{-10} M [24]. By preparing stock solutions of sodium dithionite in nitrogen-purged buffer and using the published equilibrium constants, the signal in Fig. 2A was estimated to represent approximately 2×10^{-8} M $\dot{SO}_2^-$. From the equilibrium constant and the concentration of $\dot{SO}_2^-$, the concentration of dithionite can be calculated as $\sim 6 \times 10^{-7}$ M. The dithionite absorption coefficient of 8000 $M^{-1} cm^{-1}$ at 315 nm [23] implies an absorbance of 0.005 at this concentration. Attempts to detect an increase in absorbance at 315 nm were unsuccessful, in part because of the broad absorbance of NADPH at 340 nm.

The sulfur dioxide anion radical could be detected in microsomal incubations with 1 mM sulfite (Fig. 3A). Under the same conditions no signal could be detected when the microsomal protein was replaced by either cytosolic (Fig. 3B) or mitochondrial (Fig. 3C) protein. Similar results were obtained with a higher sulfite concentration (10 mM) with correspondingly higher radical concentrations (Fig. 2A).

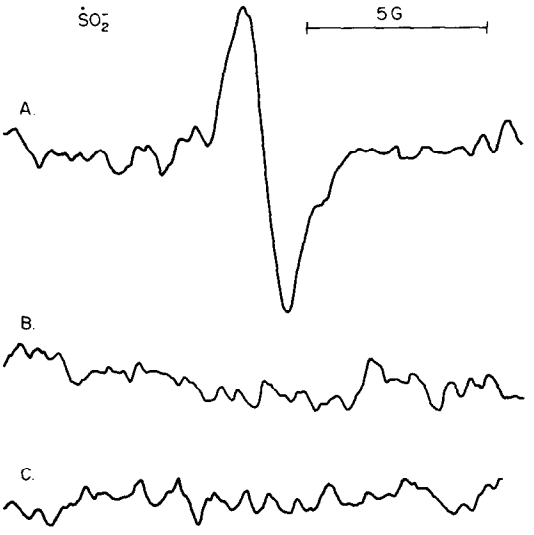

Fig. 3. Electron spin resonance spectra of incubations containing (A) 1.1 mg/ml microsomal protein; (B) 1.8 mg/ml cytosolic protein; and (C) 1.4 mg/ml mitochondrial protein and sodium sulfite (1 mM) in 0.1 M phosphate buffer (pH 7.4) and the NADPH-generating system described in Materials and Methods. Instrumental conditions were: scan time, 8 min; modulation amplitude, 0.67 G; time constant, 4.0 sec; and microwave power, 20 mW.

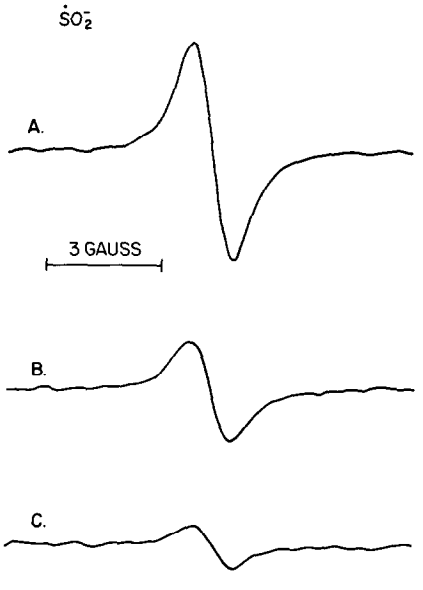

Fig. 4. Electron spin resonance spectra of incubations containing 1 mg/ml microsomal protein, sodium sulfite (10 mM) in 0.1 M Tris–HCl buffer (pH 7.4) and the NADPH-generating system described in Materials and Methods. Key: (A) the microsomal incubation was under a 100% nitrogen atmosphere; (B) identical to A, but under a 100% carbon monoxide atmosphere; and (C) identical to A, but containing 0.5 mM metyrapone. Instrumental conditions were: scan time, 4 min; modulation amplitude, 0.83 G; time constant, 2.0 sec; and microwave power, 20 mW.

If the nitrogen atmosphere (Fig. 4A) was replaced by 100% carbon monoxide (Fig. 4B), the ESR signal decreased by over 50%. The cytochrome P-450 inhibitor metyrapone (0.5 mM) also suppressed the ESR signal by 80% (Fig. 4C). These inhibitors are not radical scavengers, because incubations of dithionite (0.25 mM) alone, under a 100% CO atmosphere, or with metyrapone (5 mM) were examined for the $\dot{SO}_2^-$ ESR signal, and all three incubations contained the signal attributed to $\dot{SO}_2^-$, with no significant difference in intensities (data not shown).

When the NADPH-generating system was replaced by an NADH-generating system, the sulfur dioxide anion radical concentration was 30% lower after 3 hr. Inhibition by carbon monoxide and metyrapone also occurred, as it did with the NADPH-generating system. The radical concentration obtained when a combination NADPH- and NADH-generating system was used was not different from that obtained with the NADPH-generating system alone.

Discussion

Our results imply that cytochrome P-450 reduces (bi)sulfite (SO_2) to the sulfur dioxide anion radical. If dithionite is added to a microsomal incubation, as is done to reduce cytochrome P-450 for the standard optical P-450 assay [25], the same spectrum results. In fact, Hintz and Peterson [26] have shown that the sulfur dioxide anion radical is the actual species which reduces cytochrome P-450 when dithionite is added to microsomes. Therefore, the well known reduction of cytochrome P-450 by dithionite is reversible,

$$\text{P-450 } (Fe^{2+}) + SO_2 \rightleftarrows \text{P-450 } (Fe^{3+}) + \dot{SO}_2^-$$

$$\rightleftarrows \text{P-450 } (Fe^{3+}) + 1/2\, S_2O_4^=$$

and a redox equilibrium is established between dithionite/(bi)sulfite and NADPH/$NADP^+$, with cytochrome P-450 and NADPH-cytochrome P-450 reductase as the catalysts. Although both the NADH- and the NADPH-supported reduction of (bi)sulfite are mediated in part by cytochrome P-450, the CO- and metyrapone-insensitive reduction may be due to direct electron donation by NADH-cytochrome b_5 reductase and NADPH-cytochrome P-450 reductase respectively. Since dithionite is a much stronger reducing agent than NADPH, the formation of sulfur dioxide anion radical is the result of mass action. This reaction is unfavorable and is probably not physiologically significant under aerobic conditions.

The sulfur dioxide anion free radical is not detected in the presence of air. At least two explanations are possible for this phenomenon. First, the sulfur dioxide radical metabolite may be produced under aerobic conditions, but subsequently react with oxygen. The air oxidation of this free radical [to form superoxide and regenerate (bi)sulfite] occurs with a rate constant of $4.1 \times 10^3\ M^{-1}\ sec^{-1}$ [24]. Second, since rat hepatic microsomes in the presence of NADPH will reduce molecular oxygen to superoxide [27–29], (bi)sulfite and oxygen may be reduced at the same site(s). If oxygen is the better electron acceptor, it could prevent the reduction of (bi)sulfite by competitive inhibition. The extent of microsomal reduction of (bi)sulfite in air is unknown. The known NADPH-supported oxidation of (bi)sulfite by aerobic incubations of hepatic microsomes [9] will complicate future investigations of the possible simultaneous reduction of (bi)sulfite.

In summary, the study of the metabolism of (bi)sulfite has centered upon its oxidation to sulfate, but the enzymatic reduction of (bi)sulfite is also possible. Anaerobic incubations of (bi)sulfite with rat hepatic microsomal protein and NADPH or NADH produce the sulfur dioxide anion radical, $\dot{SO}_2^-$. The formation of this species was inhibited 50% by carbon monoxide or 80% by metyrapone, implying

that cytochrome P-450 reduces (bi)sulfite (SO_2) to $\dot{S}O_2^-$ via a one-electron transfer.

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