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ON THE MECHANISM OF THE CHROMATE REDUCTION BY GLUTATHIONE: ESR EVIDENCE FOR THE GLUTATHIONYL RADICAL AND AN ISOLABLE Cr(V) INTERMEDIATE

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With a view of elucidating the role of glutathione (GSH) in the biochemical pathways of the chromate-exposure related carcinogenesis, we carried out electron spin resonance (ESR) spectroscopic investigations of the chromate-GSH redox reactions. The ESR measurements, employing spin-traps, provide evidence for the involvement of the glutathione (GS·) radical, as well as an isolable Cr(V)-glutathione intermediate. These results indicate a new mechanism for the reduction of chromate by GSH in *in vitro* cellular environment and help understand the (unexpected) increase in Cr(VI)-induced DNA strand breaks at elevated GSH levels.

We report here indirect electron spin resonance (ESR) evidence for the involvement of the glutathionyl (GS·) radical and direct evidence for a Cr(V) intermediate in the reactions of glutathione (GSH) with chromate for the first time. This work was undertaken to clarify the biochemical pathway of the reduction of chromate by GSH, since this reduction process is thought to be a critical step in the pathogenesis of the chromate-exposure related carcinogenesis[1]. This follows from the literature reports which show that (a) Cr(VI) compounds have been proven to be carcinogens in laboratory animals as well as in human studies[2-4], (b) Cr(VI) and not Cr(III) compounds are mutagenic[5-7], (c) Cr(VI) and not Cr(III) compounds can penetrate cell membranes[8,9], (d) the final state of Cr(VI) in cellular reductions is Cr(III)[1], (e) by itself chromate (or Cr(VI)) cannot be directly mutagenic since Cr(VI) is known not to interact with isolated DNA under physiological conditions[10], suggesting the significance of Cr(VI) reduction processes. One of the major reductants in cellular

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environments is thought to be the GSH, both outside and inside the cells[11-13]. Other evidence for the role of GSH in the chromate toxicity is seen from recent studies showing that exposure of hamster cells to non-toxic levels of added selenite increases the levels of GSH as well as the Cr(VI)-induced DNA strand breaks[14], and that such DNA strand breaks in hepatocytes also change in direct proportion to their GSH content[15,16]. These observations were interpreted as implying that the reduction of chromate by GSH to some reactive intermediate is an important step in the chromate carcinogenicity[15,16]. The following reaction steps were suggested[15,16]:

(A) Initial step:

$$CrO_4^{2-} \xrightarrow[GSH]{} GSCrO_3^- \qquad (1)$$

(B) At high GSH levels:

$$GSCrO_3^- \xrightarrow[GSH]{GSSG} Cr(IV) \qquad (2)$$

(C) At low GSH levels:

$$GSCrO_3^- \xrightarrow{GS\cdot} Cr(V) \qquad (3)$$

where $GSCrO_3^-$ is the glutathione-chromate-ester, GSSG the glutathione dimer, and GS· the glutathionyl radical. Thus far, however, in these reactions, the GS· radical has eluded spectroscopic detection, perhaps because of its high reactivity, whereas both positive[17,18] as well as negative[11] evidence for Cr(V) formation has been reported. In the present undertaking, we have used the ESR spin-trap methodology to circumvent the aniticipated high reactivity of GS· and find definitive evidence for the formation of this radical. In addition, we provide unequivocal evidence for the involvement of an intermediate species containing Cr(V).

MATERIALS AND METHODS: ESR spectra were obtained at X-band (~9.7 GHz) using a Bruker ER200D ESR spectrometer. The magnetic field was calibrated with a self-tracking NMR Gaussmeter (Bruker, Model ER035M) and the microwave frequency was measured with a Hewlett-Packard (Model 5340A) frequency counter. The spin probes, α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (4-POBN) and 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) were purchased from Aldrich, and used without further purification since very weak or no spin adduct signals were obtained

from the purchased sample when used alone. $K_2Cr_2O_7$ was purchased from Fisher. All experiments were done at room temperature.

RESULTS AND DISCUSSION: An aqueous solution of 0.1 M spin trap α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (4-POBN), either with chromate alone or GSH alone, did not give a detectable ESR spectrum. However, when chromate, GSH and 4-POBN were mixed, an ESR spectrum was observed which was a composite of the spin adduct signal (sharp doublets of triplets) and that of Cr(V)[17,18], the broad peak at g = 1.995 (Fig. 1 a-c). About ten minutes later, when the signal from Cr(V) had decayed, a clear spectrum (g = 2.0061), consisting of only doublets of triplets was obtained (Fig.1 f). This spectrum is assigned to the 4-POBN-GS adduct by its strong similarity to the spectrum reported earlier[19] for the same adduct. The analysis of the spectrum in Fig. 1 f gave the nitrogen hyperfine coupling a_N = 15.0 G and proton hyperfine coupling a_H = 2.3 G, which compare well with those (a_N = 15.13 G and a_H = 2.32 G) reported earlier[19].

Additional support for this identification was seen from ESR spectra using 5,5-dimethyl-1-pyrroline-1-oxide (DMPO). The spectrum obtained, Fig. 2 a, is composite of that of the spin adduct, a 1:2:2:1 quartet, and that of Cr(V), as evidenced by the broad peak at g = 1.995. The analysis of the ESR spin adduct spectrum gave a_N = 15.2 G and a_H = 15.9 G. These values are very similar to those (a_N = 15.4 G and a_H = 16.2 G) reported[20,21] earlier for the DMPO-GS spin adduct. The spin adduct spectrum shows rapid decay essentially as decribed[20,21] previously.

An important observation in the spin-trap studies was that an increase in the amount of GSH caused an increase in the spin adduct ESR signal until the intensity leveled off at a molar ratio of about fifteen to one of GSH to $K_2Cr_2O_7$ (Fig. 1 a-c). No spin adduct ESR signal was detected for the molar ratio of less than one (Fig. 1 e). This observation is in contradiction to the steps (B) and (C) as outlined in equations (2) and (3) above.

Fig. 1 and Fig. 2 a-b provide direct evidence for the formation of a fairly long-lived Cr(V) intermediate whe the molar ratios of GSH to $K_2Cr_2O_7$ are higher than one. In agreement with earlier studies[17,18], different kinds of Cr(V) complex are formed depending

on the reaction conditions (Fig. 1 a-d). We were able to isolate the g = 1.995 species with a yield of about 50 percent. A typical ESR spectrum for the powder is shown in Fig. 2 c. The measured g-values are $g_{\parallel}$ = 2.007 and $g_{\perp}$ = 1.989, with little variation with temperature from 115 K to 310 K. These values are typical of Cr(V) solids[22]. When the isolated Cr(V) intermediate was redissolved in water, its ESR spectrum was identical with that from the original solution.

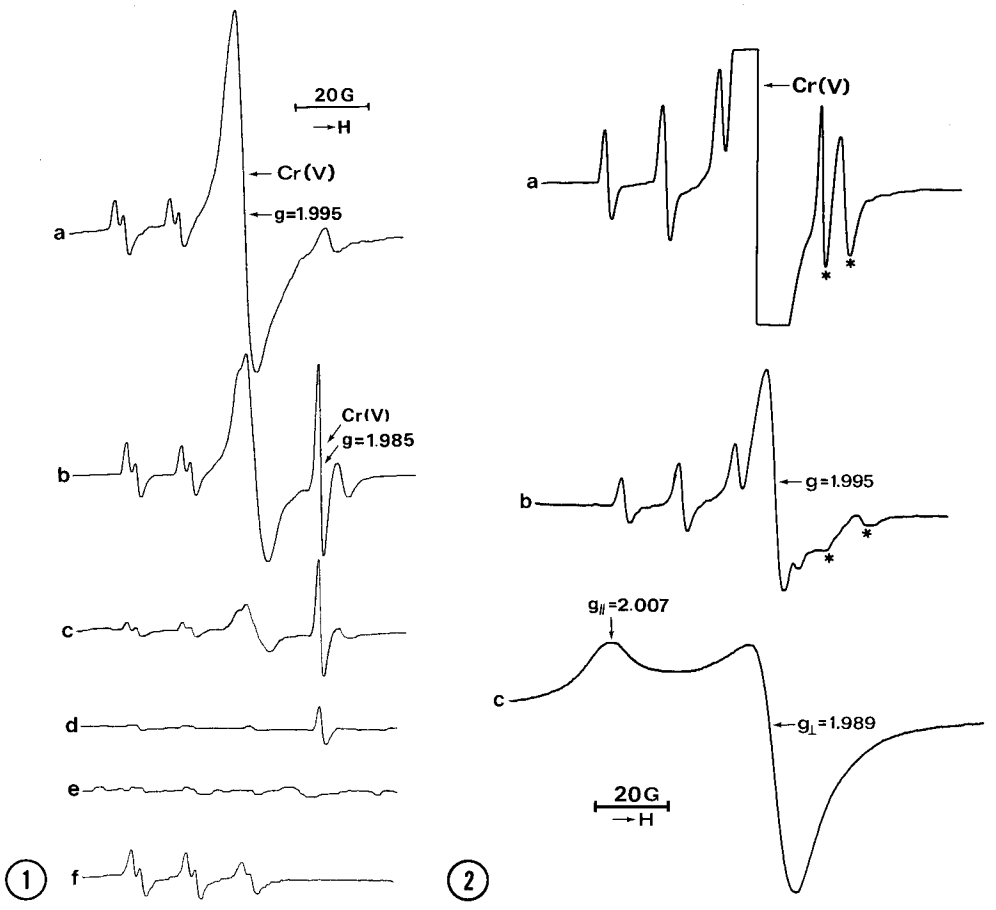

Fig. 1. ESR spectra recorded 2 minutes after mixing a 0.015 M solution of $K_2Cr_2O_7$ with the following concentrations of GSH: (a) 0.375 M, (b) 0.15 M, (c) 0.075 M, (d) 0.03 M, (e) 0.0075 M. The spectrum in (f), corresponding to [GSH] = 0.575 M, was taken after 10 minutes of the mixing, in order to obtain the spin adduct signal free of the Cr(V) signals. The concentration of spin trap used, 4-POBN, was 0.1 M.

Fig. 2. ESR spectra of chromate-glutathione mixtures: [$K_2Cr_2O_7$] = 0.015 M; [glutathione] = 0.375 M; [DMPO] = 0.1 M; (a) pH = 7.2; (b) pH = 4.0 M, recorded 2 minutes after mixing. (c) ESR spectrum of an isolated Cr(V) complex. The astrisks indicate minor Cr(V) species.

Using the isolated Cr(V) complex, as well as K_3CrO_8, a known Cr(V) compound[22], it was found that the Cr(V) intermediate also serves as an oxidant for the formation of GS·, a result not discussed in any of the earlier studies of the chromate-GSH reaction[1,8-18]. We thus suggest the following alternative mechanisms for the chromate reduction:

(A) At high GSH levels:

$$Cr(VI)(GS) \xrightarrow[GSH]{} Cr(V)GS + GS\cdot \quad (4)$$

$$Cr(V)GS \xrightarrow[GSH]{} Cr(V)(GS)_2 \quad (5)$$

$$Cr(V)(GS)_2 \xrightarrow[GSH]{} Cr(III)GS \quad (6)$$

$$2GS\cdot \longrightarrow GSSG \quad (7)$$

(B) At low GSH levels:

$$Cr(VI)GS \xrightarrow[GSH]{} Cr(IV) + GSSG \quad (8)$$

$$Cr(IV) + Cr(VI) \xrightarrow[6GSH]{} 2Cr(III)GS + 2GSSG \quad (9)$$

The above results also help understand the recent reports[15,16] that increased levels of GSH in the cells result in increased DNA damage by Cr(VI). In the cellular environment the concentration of GSH is much higher than that of Cr(VI)[10]. Now this study shows that the simultaneous formation of GS· and Cr(V) takes place only at higher levels of GSH relative to Cr(VI). It thus appears that increased Cr(VI)-induced DNA strand breaks noted at higher GSH levels are related to the simultaneous formation of the GS· radicals and Cr(V) intermediates.

In conclusion, this work shows that both a long-lived, isolable, Cr(V) intermediate, and the GS· radical are formed in the reaction between chromate and GSH, but only at high levels of GSH relative to chromate ions, in disagreement with an earlier proposed mechanism[15,16]. Thus, a new mechanism has been suggested, which also provides a plausible explanation for the recent (unexpected) report of an increase in Cr(VI)-induced DNA strand breaks at increased GSH levels. Our success in isolating the Cr(V) complex opens up the

possibility of carrying out a detailed single crystal characterization of $Cr(V)-(GSH)_n$ complexation, which may provide clues as to the mechanism of the chromate reduction by cellular thiols. Also a methodology for combating chromate-related carcinogenesis may be available as a results of these investigations.

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