The Isolation and Characterization of a Chromium(V) Containing Complex from the Reaction of Glutathione with Chromate

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Abstract

A material which analyses as $Na_4Cr(GSH)_4 \cdot 8H_2O$ can be reproducibly precipitated from the reaction of glutathione with chromate. Spectroscopic evidence suggests that this is predominantly a chromium(V) complex of glutathione, involving carboxylate and thiolate coordination to the metal.

Introduction

There is considerable current interest in the molecular species involved in the carcinogenicity and mutagenicity of chromium(VI) [1, 2]. It is generally accepted that the chromate ion, CrO_4^{2-}, the dominant form of chromium(VI) in neutral aqueous solutions, can readily cross cellular membranes via non-specific anion carriers [1]. Studies of model systems support the suggestion of a facile uptake mechanism for chromate [3–5] and the widespread use of ^{51}Cr labelled chromate to tag erythrocytes [6] is based on the fact that once within the cell chromium, in a reduced form, is immobilized. In contrast, it is in general difficult for chromium(III) complexes to enter cells, although certain ligands may greatly facilitate uptake [7].

In vitro experiments on chick embryos hepatocytes have shown that glutathione (GSH) [8] could have a potentiating effect on the toxicity of chromate; an increase in the number of DNA strand breaks was observed in cells in which GSH had been induced. There is now a considerable amount of evidence that a number of reactive species, including thionyl radicals as well as chromium(V) complexes, can be generated during the reduction of chromium(VI) by GSH [9–11]. Reactive intermediates may cause strand breaks in DNA by a variety of mechanisms, including pathways involving the hydroxyl radical [10, 11]. However, both *in vivo* and *in vitro*, chromium(VI) can damage DNA in a number of other ways such as by causing intrastrand cross-links and by cross-linking to proteins [12, 13].

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There are now many papers which report the generation of chromium(V) complexes during the reduction of chromate by glutathione [9–11, 14–18]. We were the first to report the generation of a chromium(V) species during the reaction of chromate with glutathione at room temperature [14] and observed a species characterized by $g = 1.996$. Subsequently, using lower GSH concentrations, other workers have observed another chromium(V) species, characterized by a g value close to 1.985 [9–11, 15–18], as well as the $g = 1.996$ species. Chromium(V) complexes may be important intermediate species in mediating the damage caused to DNA by chromate.

We have previously mentioned [18] that a solid material with $g = 1.996$ can be isolated from the reaction of sodium chromate with excess glutathione. On dissolution this substance is able to cause strand breaks in PM2 DNA [19]. The increased interest in chromate toxicity [9, 12, 16] and the role of chromium(V) species and other reactive species in mediating chromate toxicity [9–11], prompts us to report full details of the preparation and properties of the chromium(V) intermediate we have isolated from the Cr(VI)/GSH system. Although this complex is unlikely to be formed *in vivo* (as it is formed at high concentrations of GSH) we believe it is a convenient source of reactive intermediates capable of damaging DNA directly; such species may well be formed *in vivo*.

Results and Discussion

Properties of the Solid Complex

The solid compound was isolated by the rapid addition of methanol to a cold solution of chromate and glutathione. The compound separated as a light green amorphous, powder, readily soluble, with decomposition, in water or water alcohol mixtures. Considering that the complex was isolated from a reacting mixture, and is an unstable intermediate, remarkably consistent microanalysis results were obtained. Results are summarized in Table 1, and suggest an empirical formula close to $Na_4Cr(GSH)_4 \cdot$

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TABLE 1. Analytical results[a]

	Calculated	Sample no. 1	Sample no. 2	Sample no. 3
Cr	3.45	3.61(n)	3.50(n)	3.35(c)
		3.64(a)	3.71(a)	
Na	6.01	6.1	6.1	
C	31.85	31.08	31.01	
H	5.01	4.97	4.57	
N	11.15	10.90	10.02	
S	8.49	8.55		
H_2O	9.56	9.50		
g_{av}		1.996	1.996	1.996
μ_B		2.4(±0.3)		2.5(±0.3)

[a]The analytical results presented above can be interpreted in terms of an empirical formula involving four moles of glutathione and one chromium atom $Na_4Cr(GSH)_4 \cdot 8H_2O$ ($CrNa_4C_{40}H_{75}N_{12}S_4O_{32}$, RMM = 1508 or an equivalent formulation involving GSSG). Chromium analysis by atomic absorption; n, neutral solution; a, acid solution; c, using 1,5-diphenylcarbohydrazide in neutral solution.

$8H_2O$, although some of the glutathione may be present in the oxidized form (i.e. as GSSG) *vide infra*.

The dominant form of chromium in the mixture can readily be confirmed as chromium(V) by the relatively sharp EPR spectrum, $g = 1.996$ obtained at room temperature (Fig. 1). Integration of the solution spectrum of the complex in aqueous solution and extrapolation to zero time [20] supports this suggestion. The electronic spectrum in the visible region is dominated by a broad band centred at 640 nm, with a shoulder at 900 nm (Fig. 1). The infrared spectrum of the solid intermediate is not particularly informative. The antisymmetric carboxylate stretch is at 1640 cm^{-1} and there is no obvious S–H stretch (*c.* 2540 cm^{-1}).

The magnetic moment of the material was always slightly greater than that expected for a d^1 complex; typical values are given in Table 1. There are two factors influencing the value observed.

(i) The error on the measurement is large (up to 0.3 μ_B due to the large diamagnetic contribution from ligands with a high relative molar mass).

(ii) There is probably some contamination with a chromium(III) or another paramagnetic species, which cannot be removed by the preparation procedure here used.

A relatively small amount of a low molecular weight chromium(III) impurity would have a marked effect on the magnetic moment observed. Also, it is known, *vide infra*, that on dissolution in water the complex can generate thionyl radicals [16], so contamination with a radical species could also be responsible for the high magnetic moments observed. Significant contamination with chromate can be ruled out as no chromate can be detected in the infrared

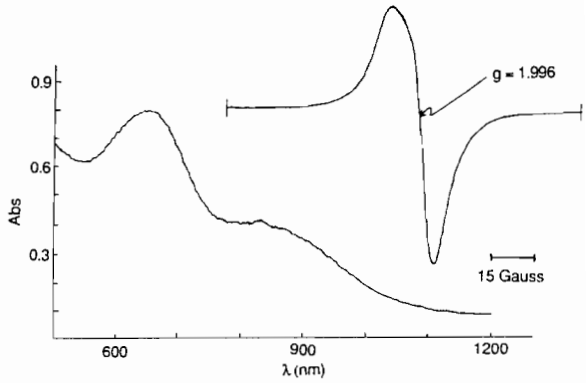


Fig. 1. Electronic and EPR spectrum of the solid isolated from the reduction of chromate with glutathione.

spectra. The magnetic moment of the complex is constant within experimental error over the liquid nitrogen temperature range, which supports the formulation of the complex as a monomeric chromium(V) species. The reactive nature of the compound makes purification impossible and although we have investigated a range of precipitation procedures the method reported here reproducibly leads to the precipitation of the dominantly chromium(V) containing material described in this paper. Present investigations are being carried out over a wide range of reaction conditions and solvents to see if a purer compound can be isolated. However, the widespread interest in this material and its ability to produce strand breaks in PM2 DNA [19] lead to this report of its properties at this stage. The above results suggest that the complex is polymeric (on the grounds of its insolubility in methanol) and that coordination involves deprotonated thiolate and carboxylate functions.

Properties in Aqueous Solutions

On dissolution in water the complex decomposes to chromium(III) containing species. The rate of disappearance of the complex has been followed at 650 nm (in water or GSH, pH 7.0, 25 °C), the decomposition is first order and the rate is retarded by low concentrations of GSH and then catalysed by higher concentrations (Fig. 2). This observation is consistent with the kind of mechanism outlined in our earlier work [20], i.e. glutathione both acts as a complexing agent stabilizing chromium(V) and as a reducing agent. The rates are similar to those we have calculated from observations on the reaction of GSH with chromate, e.g. in 0.3 mol dm^{-3} GSH, Fig. 2, k_{obs} is *c.* 6×10^{-3} s^{-1}, compared to 4.75×10^{-3} s^{-1} from ref. 20.

In the absence of added glutathione chromate ($\lambda_{max} = 375$ nm) is formed during the reaction; this can clearly be seen in the electronic spectrum of reaction mixtures allowed to stand for several hours (Fig.

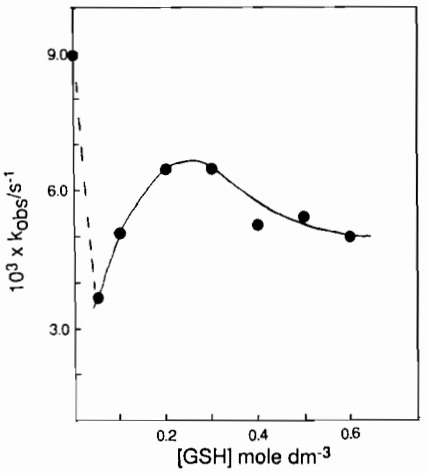


Fig. 2. Variation of the pseudo first order rate constants for the disappearance of the intermediate with glutathione concentration (pH = 7.0, 25 °C, followed at 650 nm).

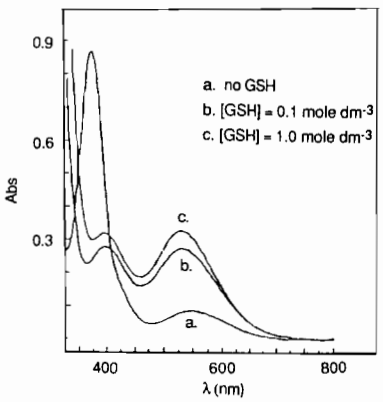


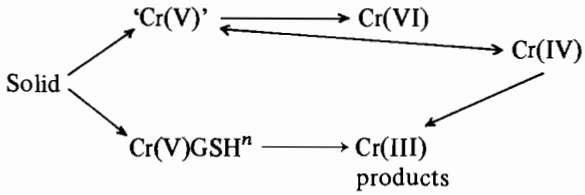
Fig. 3. Electronic spectra of the green intermediate in aqueous solution and the final chromium(III) complex under different reaction conditions. Total concentration of chromium in the initial solution: (a) 1.29×10^{-3} mol dm^{-3}; (b) 1.28×10^{3} mol dm^{-3}; (c) 1.27×10^{-3} mol dm^{-3}; pH = 7, 22 °C.

3) and typically accounts for about 50% of the chromium in the original complex (53% from two duplicate spectrophotometric determinations of chromate, reproducible to within 5% relative). This observation suggests that the complex should be formulated as a complex of GSH and GSSG, i.e. $Na_4CrGSH_2GSSG \cdot 8H_2O$. This formulation is further supported by two further independent measurements.

(i) In solutions of the green intermediate allowed to stand in water for 2 h there is no unreacted glutathione (by Ellman's method).

(ii) The reduction of chromate is essentially complete, over 2 h in a solution of sodium chromate [Cr]:[GSH], 4:1 ($[CrO_4^{2-}] = 1 \times 10^{-3}$ mol dm^{-3}, pH = 7).

The final chromium(III) containing product of the reaction in the presence of excess glutathione has a circular dichroism ($\Delta\epsilon_{Cr,580} = -0.54$ cm^{-1} mol^{-1} dm^3, in 1 mol dm^{-3} glutathione) and electronic spectrum ($\epsilon_{Cr,545} = 255$, $\epsilon_{Cr,395} = 251$ cm^{-1} mol^{-1} dm^3) similar in energy to, but more intense, than those we have recently reported for a chromium(III) GSSG complex ($\Delta\epsilon_{Cr,580} = -1.94$, $\epsilon_{Cr,545} = 59.5$, $\epsilon_{Cr,395} = 68$) [21]. A scheme, consistent with our earlier suggestions concerning this reaction, and capable of explaining all the above observations is given below (Scheme 1).



Scheme 1. Reaction scheme for the decomposition of the intermediate. Some of the potential pathways from the solid glutathione intermediate to chromium(III); note that the chromium(III) products produced by the various pathways could be different.

On dissolution in water the complex generates significant quantities of both the $g = 1.996$ and the $g = 1.986$ chromium(V) species, but when the complex is dissolved in 0.5 molar GSH only the $g = 1.996$ species predominates (Fig. 4). When the complex is

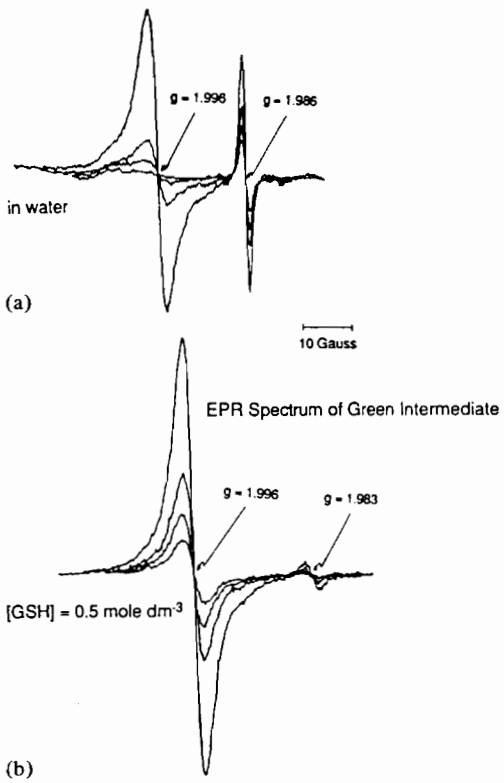


Fig. 4. EPR spectrum of the intermediate in aqueous solution: (a) in water times after mixing, 240, 320, 380 and 460 s; (b) in 0.5 mol dm^{-3} glutathione, pH = 7.0, 22 °C, times after mixing, 140, 180, 220 and 250 s.

incubated with the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), with or without added glutathione, a spectrum typical of the trapped glutathionyl radical can be observed [12, 13, 16].

The initial electronic spectrum of the complex in aqueous solution has been calculated by extrapolating the first order decay, at various wavelengths, to zero time. The dominant absorption of the complex is at 650 nm (ϵ_m 1040 cm^{-1} mol^{-1} dm^3) similar to the intermediate we reported in an earlier paper [20]. The circular dichroism of the complex in aqueous solution (obtained as a rapid scan) shows two bands, a minimum at 630 nm and a maximum at 880 nm; these correspond to the absorptions observed for the solid complex (Fig. 1). This kind of spectrum is typical of that observed for a low-symmetry d^1 complex in which the $^2T_{2g}$ state is split [22].

Conclusions

A material which analyses as $Na_4Cr(GSH)_4 \cdot 8H_2O$ can be reproducibly precipitated from the reaction of glutathione with chromate. Spectroscopic evidence suggests that this is predominantly a chromium(V) complex of glutathione, involving carboxylate and thiolate coordination to the metal. The material is a convenient source of reactive intermediates and may be stored for considerable lengths of time; consequently it may be of interest to those wishing to study an alternative model of chromate toxicity *in vitro*.

Experimental

Caution: chromate is a known mutagen and carcinogen, the chromium(V) complex described in this paper is known to cause strand breaks in DNA [19] and should be handled with suitable precautions.

Materials

Sodium dichromate was BDH AnalaR grade; glutathione and dmpo were purchased from Sigma; all other chemicals were purchased from BDH Chemicals.

Methods

Electronic spectra were measured with a Perkin-Elmer 330, circular dichroism with a JASCO J300 instrument and infrared spectra as either nujol mulls or KBr discs (1%, 200 mg) between 4000 and 400 cm^{-1} using a Mattson Polaris FTIR. Kinetic measurements were made in water or solutions of glutathione at pH = 7.0, 25 °C, with no added electrolyte. EPR spectra were recorded with a Bruker ERD/2000/10 instrument, immediately on dissolution or in the spin trapping experiments 3–10 min after mixing a 0.1 mol dm^{-3} of dmpo (*c.* 5 cm^3) with 5–10 mg of the solid chromium(V) complex, as previously described [23]. Magnetic susceptibilities were measured on a Faraday balance described previously [24]. Microanalyses were done by the University College, London Laboratories, or Butterworths Laboratories; chromium was determined by atomic absorption (after acid or aqueous dissolution of the complex, Butterworths), or as the 1,5-diphenylcarbohydrazide [25].

Synthesis of the Complex

Ice cold solutions of glutathione (5 cm^3, 1.0 mol dm^{-3}, pH 7.0 ± 0.1) and sodium chromate (5 cm^3, 0.1 mol dm^{-3}), were mixed and allowed to react at *c.* 0 °C for 150 s. A large excess (60 cm^3) of methanol was then added, causing a green precipitate to form almost immediately. The reaction mixture was stored in a deep freeze, at –25 °C, for 1–48 h and the product removed by filtration and dried *in vacuo* over P_2O_5.

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