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Mechanisms in the Reduction of Chromium(VI) with Glutathione

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Abstract

The reduction of chromate by glutathione (GSH), at neutral pH, has been studied by following the generation and decay of a green intermediate (λ_{max} = 650 nm) believed to be a chromium(V) complex. The formation of the intermediate follows a rate law

$$k_{1\,obs} = 0.35(\pm 0.1) \times [GSH]^2\ s^{-1}$$

and its disappearance a rate law

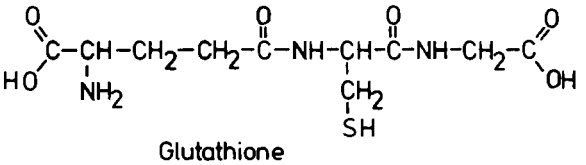
$$k_{2\,obs} = 1.45(\pm 0.2) \times 10^{-3} + 9.1(\pm 0.6) \times [GSH] \times 10^{-3}\ s^{-1}$$

(20 °C, pH = 7.0). The results of this study are compared with earlier studies in which buffer was used (Tris); buffers, in general, alter the course of the reduction of chromate by glutathione. Chromium(V) intermediates are not generally observed in solutions buffered with Tris, and Tris may also catalyse the rate determining reduction of the chromium(VI) thiolate ester.

Introduction

There is considerable current interest in the carcinogenicity and mutagenicity of chromium(VI) [1, 2]. The chromate ion, CrO_4^{2-}, the dominant form of chromium(VI) in neutral aqueous solutions, can readily cross cellular membranes via nonspecific anion carriers [1]. Detailed 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 [5].

Partially reduced species generated from chromate within the cell are probably the active toxins *in vivo*; and the two step process leading to toxicity has been termed the 'uptake-reduction' model [1]. *In vitro* experiments on chick embryos suggest [7] that glutathione (γ-glutamylcysteinylglycine, GSH (I)) may have a potentiating effect on the toxicity of chromate *in vivo*. We [8], and others [9], have shown that chromium(V) species can be generated from the reaction of GSH with chromate at neutral pH. Chromium(V) can also be generated in the reaction of chromate with a variety of other cell components such as mitochondria [10, 11].



Glutathione

We have been studying the complexes formed by glutathione and chromium for a number of years [8, 12], and have become interested in discovering if the ultimate genotoxic form of chromium is an intermediate chromium complex involving glutathione, in one of the oxidation states ((V), (IV) or (III)), potentially formed after the initial absorption and reduction of chromate. We are particularly interested in a hypothesis in which genotoxicity is expressed by a relatively stable chromium(V) species. However, another possibility is that DNA is damaged by the *in vivo* generation of hydroxyl radicals from some reaction involving GSH, chromium in one of its higher oxidation states and molecular oxygen.

Studies of the formation of strand breaks in DNA may help in assessing these various hypotheses. Two independent studies have shown that the combination of hydrogen peroxide and chromate can produce strand breaks in double strand DNA [13, 14]. Work from our laboratories has shown that a combination of chromate and GSH in the absence of H_2O_2 can cause strand breaks in the supercoiled circular DNA of bacteriophage PM2 [15]. Other workers have detected little or no DNA damage in the absence of hydrogen peroxide [14, 15]; this may be because the sensitivity of various assays based on supercoiled DNA varies.

In this paper we wish to report some of our studies of the reaction of glutathione with chromate. Two aspects of this chemistry are of interest; firstly any effects of reaction conditions, especially buffer, on the pathway for the reduction of chromate,

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and secondly the role of chromium(V) complexes in this reaction in relation to the green intermediate chromium species we have previously isolated from this system [16].

There have been a number of studies of the reaction of chromate with glutathione and related thiols (RSH). McAuley and Olatutunji first studied the CrO_4^{2-}/GSH system [17]; they concluded that the reaction proceeded in acidic solution via the initial formation of a thiolate ester species (**1**) (in preequilibrium), and followed an overall rate law

$$-d[Cr(VI)]/dt = K[RSH](k_a[H^+]^2 + k_2[RSH][Cr(VI)])/1 + K[RSH]$$

Connett and Wetterhahn have followed up this work with studies of the reactions of several thiols with chromate in neutral solution [1, 18, 19]. The reaction of glutathione with chromate is unusual in that it proceeds in two distinct steps. Other thiols, in an excess, generally lead to a simple first order disappearance of chromate. However, various other reducing agents e.g. lactate also have a complicated multistep mechanism for the reduction of chromate [20]; in the case of lactate this involves the formation of considerable quantities of chromium(V).

In the present study we have carefully investigated the reaction of chromate with glutathione in the absence of buffer. Our results point to a more complicated series of reactions for this system than has previously been appreciated.

Results

Chromate and Glutathione (no buffer)

In a preliminary communication [8] we drew attention to the apparently simultaneous disappearance of a chromium(V) EPR ($g = 1.996$) signal and a green intermediate complex formed in the reaction of glutathione and chromium(VI). Goodgame and Joy [9] subsequently observed a similar EPR signal ($g = 1.996$) in the presence of excess glutathione. We have since successfully isolated a chromium complex from these solutions and details of the preparation and properties of this compound will be provided in a later paper; a preliminary communication concerning this compound has appeared [16].

As previously reported, on mixing solutions of glutathione and chromate (pH = 7.00, 20 °C, $[CrO_4^{2-}] = 1 \times 10^{-3}$ mol dm^{-3}) at concentrations of GSH greater than *c.* 0.05 molar a distinct green colour developed. The intermediate generated has a maximum absorbance close to 650 nm. These green solutions of GSH and chromium subsequently decay to give a much less intense purple colour characteristic of chromium(III).

We have now studied the course of the reaction between GSH and chromate by following the absorbance change at 650 nm. We believe that buffers and other ligands in the solution may interfere with this reaction and have consequently conducted our initial study in the absence of any buffer or extraneous ions; in all cases we have used a pseudo first order excess of glutathione and there were no significant pH changes during the course of the reaction. This is not surprising as glutathione will act as a buffer at this pH.

The rate of disappearance of the green species eventually became first order and an estimate of the rate of disappearance of this species can be obtained from plots of $\ln(A_t - A_\infty)$ for the final stages of the reaction. In general pseudo first order rate constants were obtained by a nonlinear least-squares procedure fitting the absorbance versus time curve directly to a model for consecutive first order reaction [21, 22]

$$A \longrightarrow B \longrightarrow C$$

with pseudo first order rate constants $k_{1\,obs}$ and $k_{2\,obs}$. Results of nonlinear fitting are summarized in Table 1. Typical fits of plots of A_{obs} versus time are shown in Fig. 1.

At the higher concentrations of glutathione used the rate of appearance of the intermediate is too rapid to be determined by our conventional methods ($t_{0.5} = c.$ 10 s). For these concentrations we only report the value of $k_{2\,obs}$ as determined from plots of $\ln(A_t - A_\infty)$ versus time.

TABLE 1. Pseudo first order rate constants for the appearance and disappearance of green intermediate

[GSH] (mol dm^{-1})	$10^2 \times k_{1\,obs}$ (s^{-1})	$10^3 \times k_{2\,obs}$ (s^{-1})
0.1	0.409(0.024)	1.75(0.03)
	0.451(0.025)	1.81(0.03)
0.2	1.72(0.058)	2.92(0.16)
	1.51(0.11)	3.27(0.16)
0.3	3.22(0.20)	4.51(1.8)
	3.21(0.22)	4.75(0.21)
0.4	5.62(0.32)	5.34(0.14)
	5.54(0.36)	4.74(0.15)
0.5	7.97(0.43)	6.24(0.15)
	6.81(0.23)	5.20(0.07)
		6.16
0.6		6.78
0.7		7.51
0.8		8.61
		8.67
1.0		10.5

pH = 7.0(±0.02), 20 °C, $[CrO_4^{2-}] = 1.0 \times 10^{-3}$ mol dm^{-3}.

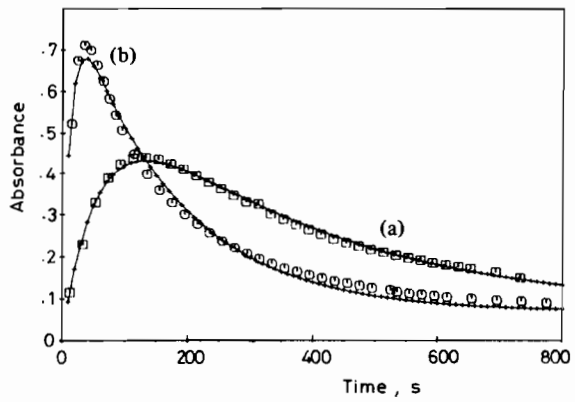

Fig. 1. Typical non-linear fits of A_{obs} (650 nm) vs. time. Fitted using (a) [GSH] = 0.2 mol dm^{-3}, $k_{1\,obs} = 1.72 \times 10^{-2}$ and $k_{2\,obs} = 2.92 \times 10^{-3}$; (b) [GSH] = 0.5 mol dm^{-3}, $k_{1\,obs} = 7.97 \times 10^{-2}$ and $k_{2\,obs} = 6.23 \times 10^{-3}$ (pH = 7.0, 20 °C, $[CrO_4^{2-}] = 1 \times 10^{-3}$ mol dm^{-3}). The fit is quite poor at the higher GSH concentration, we attribute this to errors associated with the very rapid nature of the first step of the reaction. Only values of $k_{1\,obs}$ at [GSH] < 0.4 mol dm^{-3} were used in determining the functionality of $k_{1\,obs}$.

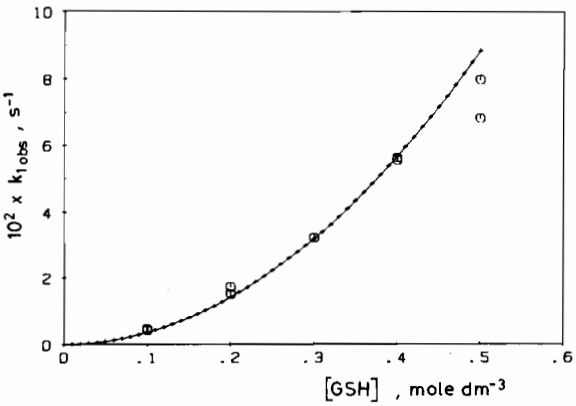

Fig. 2. Dependence of $k_{1\,obs}$ on glutathione concentration. The solid line is a least-squares fit to $y = ax^2$.

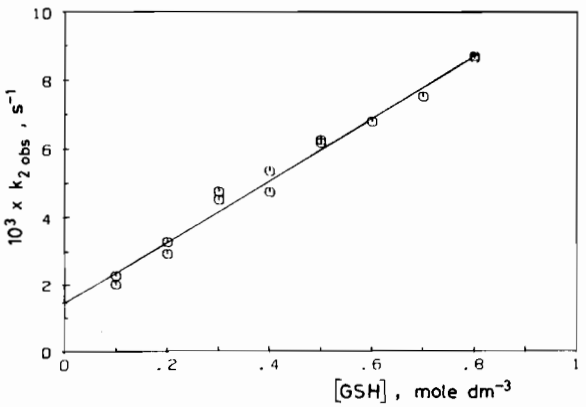

Fig. 3. Dependence of $k_{2\,obs}$ on glutathione concentration. The solid line is a least-squares fit to $y = a + bx$.

The variation of these two pseudo first order rate constants with the concentration of glutathione is illustrated in Figs. 2 and 3. The rate of formation of the intermediate appears to follow a rate law $k_{1\,obs} = a[GSH]^2$; the solid line on Fig. 2 is a least-squares fit to this equation. Attempts to fit more complicated expressions to these results (e.g. $k_{1\,obs} = a[GSH]^2/1 + b[GSH]$ as used by other workers) produced meaningless fits in which one parameter was essentially undetermined. Moreover, a nonlinear fit to $y = a[GSH]^n$, gave n values close to 2 (1.8 ± 0.2); we have hence chosen the physically more meaningful model of a reaction second order in glutathione. The rate of disappearance of the intermediate follows a simple first order rate law with an additional term independent of the concentration of glutathione ($k_{2\,obs} = a + b[GSH]$).

In a separate series of experiments we have studied the disappearance of the chromium(V) EPR signal in similar solutions of chromate and GSH. The conditions of these experiments are slightly different in that it is not possible to properly thermostat the samples in the EPR spectrometer. Given this limitation the final rate of disappearance of the ($g = 1.996$) chromium(V) EPR signal is first order and very similar to the rate at which the green intermediate disappears, e.g. $k_{obs} = 7.5 \times 10^{-3}$ s^{-1} ([GSH] = 0.8 mol dm^{-3}, room temperature, *c.* 18 °C, pH = 7.0) by EPR, compared to 8.6×10^{-3} s^{-1} measured from the electronic spectra (Table 1). Experiments in which the green intermediate we have isolated from this system [16] ($g = 1.996$) was dissolved in aqueous solution and the EPR signal integrated and extrapolated to zero time indicate that at least 80% of the chromium in this species is initially present as chromium(V); given that this is a difficult experiment, chromium(III) produced during the decomposition of the compound will broaden the signal, this is good evidence that the green intermediate is a chromium(V) species.

We believe that these observations add weight to our suggestion that the species absorbing at 650 nm is a chromium(V) complex stabilized by glutathione.

Discussion

There is an ever increasing number of studies which draw attention to the importance of intermediate oxidation states during the reduction of chromium(VI). In the context of the present study, two recent papers on the reaction of chromate with lactate [20] and oxalate [23] are of particular interest; both of these papers indicate that large quantities of chromium(V) can be generated during the reduction of chromate and that even reducing ligands such as oxalate and lactate can stabilize chromium(V) intermediates.

In modelling the reduction of chromate we will make the assumption that in aqueous solution chromium(IV) species are of low stability and unlikely to be present other than as transient intermediates (best described by a stationary state model). In the scheme we consider the initial reaction of chromate and glutathione to be the formation of the thiolate ester species (1). Rapid changes in absorbance at 380 nm observed in several studies of the reactions of thiols, and the present work, support this suggestion. This species is then postulated to undergo reduction by another mole of glutathione (2) to generate chromium(IV), which subsequently disproportionates to generate chromium(V) and chromium(III) species (3). Acid catalysed reactions, leading to the one electron reduction of chromium(VI), as observed by McAuley and Olatutunji [17] are unlikely in neutral solutions.

We shall first consider the rate of appearance of chromium(V); this appears to be second order in glutathione. A scheme is outlined below:

$$Cr(VI) + GSH \longrightarrow Cr(VI)\text{–}SG + H^+ \quad K(k_1/k_{-1}) \quad (1)$$

$$Cr(VI)\text{–}SG + GSH \longrightarrow Cr(IV) + GSSG + H^+ \quad k_2 \quad (2)$$

$$2Cr(IV) \longrightarrow Cr(V) + Cr(III) \quad k_3 \quad (3)$$

This is similar to the kind of mechanism proposed by Connett and Wetterhahn in their studies of a wide range of thiols [1, 18, 19] and that used by Wong and Pennington [24] in discussing the reduction of chromate by cysteine. Taking a preequilibrium in the thiolate ester and a stationary state in chromium(IV) we can derive the following expression for the rate of appearance of chromium(V):

$$\text{rate} = Kk_2[GSH]^2[Cr(VI)]/2 + 2K[GSH]$$

We are probably in the limit that $2K \gg 2$ (K *c.* 20 [18]) this rate law becomes first order in glutathione. One possible explanation for the experimentally observed rate law is that the rate determining electron transfer (2) is general base catalysed, with glutathione being the only general base present in significant concentration. Equation (2) can be rewritten as

$$Cr(VI)\text{–}SG + 2GSH \longrightarrow Cr(IV) + GSSG + H^+ + GSH \quad (2')$$

This would produce a rate law second order in glutathione. In earlier work [18], in which the disappearance of chromate was followed and no intermediates were observed, the buffer Tris was present in one molar concentration, thus providing a constant and substantial excess of a general base.

The disappearance of the chromium(V) spectrum follows a rate law first order in glutathione with a distinct intercept. A scheme which could account for this observation is given below:

$$Cr(V) + nL \longrightarrow Cr(V)L_n \quad \text{fast} \quad (4)$$

$$Cr(V)L_n + GSH \longrightarrow Cr(V)L_n\cdot GSH \quad K' \quad (5)$$

$$Cr(V)L_n\cdot GSH \longrightarrow Cr(III) \quad k_6 \quad (6)$$

$$Cr(V)L_n \longrightarrow Cr(III) \quad k_7 \quad (7)$$

$$\text{rate} = -k_7[Cr(V)L_n] - k_6K'[GSH][Cr(V)L_n]/1 + K'[GSH].$$

A ligand captures chromium(V) to form an intermediate (4) (ligand exchange reactions at the d^1 chromium(V) centre are likely to be extremely rapid). In this context the ligand (L) could represent carboxylate functions on GSH or GSSG or in other workers experiments a molecule of buffer. The intermediate complex then formed may decompose either by reaction with a further molecule of glutathione, postulated above as involving a rapid preequilibrium ($1 \gg K'$), to give a term first order in glutathione, and also by a path independent of any added glutathione (k_7). The fact that there is a glutathione independent path supports our suggestion [8, 16] that the intermediate is a complex of chromium(V) with a reducing agent (i.e. glutathione). Clearly if chromium(IV) were generated from the reduction of chromium(V) this could also disproportionate to generate chromium(V): although we note this possibility we shall not consider it further. Values derived using this model for interpreting $k_{1\,obs}$ and $k_{2\,obs}$ values are reported in Table 2.

TABLE 2. Rate constants derived from fits of $k_{1\,obs}$ and $k_{2\,obs}$

$k_{1\,obs} = 0.35(\pm 0.1) \times [GSH]^2\ s^{-1}$
$k_{2\,obs} = 1.5(\pm 0.2) \times 10^{-3} + 9.1(\pm 0.6) \times [GSH] \times 10^{-3}\ s^{-1}$

$k_{1\,obs}$ fitted by least-squares to $y = ax^2$ and $k_{2\,obs}$ to $y = ax + b$.

The results in Table 2 suggest a value of 0.7 mol^{-2} dm^6 s^{-1} the second order rate constant for the rate determining electron transfer process (pH 7.0, 20 °C, no ionic strength control). This value may be compared with the value of 0.2 (±0.04) reported by Connett and Wetterhahn [18] (25 °C, Tris–HCl 1 mol dm^{-3}); given the very different methods used to determine these rate constants this probably constitutes quite good agreement. The similarity in our results suggests that although Tris may both prevent the observation of chromium(V) complexes,

by forming an unstable intermediate (either a chromium(V) complex or a ternary complex involving GSH), and also act as a general base catalysing the reduction, the rate determining step, the initial reduction of the chromium(VI) thiolate ester, is the same or similar in both cases. In related work Goodgame and Joy have presented evidence for ternary Cr(V)/Ascorbate/Tris species in the reduction of chromate by ascorbate [25].

Another possibility is that the reaction may well also go via different pathways at the different glutathione concentrations. EPR studies [8, 9, 26–28] suggest that at high concentrations of GSH the reaction proceeds predominantly via the chromium(V) $g = 1.995/6$ species. It is interesting to note that the results of Kitagawa *et al.* [27] suggest that the maximum intensity of the $g = 1.996$ peak varies as the square of the GSH concentration*; an empirical observation which may well relate to our results.

The results in this paper confirm that during the reaction of GSH with chromate at neutral pH considerable quantities of chromium(V) can be formed. The stability of chromium(V) species may be affected by buffers such as Tris. The pathway of the reaction seems to depend on the GSH concentration and at the higher concentrations of GSH used in our study we see stabilization of a chromium(V) species by GSH. Preliminary experiments in our laboratories also indicate [15] a marked dependence of the rate of chromate reduction on the buffer used. This is not surprising in view of the fact that chromium(VI) is well known to form complexes with buffers such as phosphate [29].

An important species stabilizing chromium(V), in our work, may be a glutathione. More complicated reaction schemes are needed to interpret the reduction of chromate by GSH than has previously been realized, however our results are consistent with the mechanisms generally accepted for the reduction of chromate by thiols [17–19, 24]. Although the species formed at high GSH concentrations may not be of direct relevance to the *in vivo* reduction of chromate, our work is important for a full understanding of the complicated chemistry of the GSH/chromate system. Some of the intermediates generated may be important in the expression of chromate toxicity; further studies of this system are in progress.

Experimental

Materials

Potassium dichromate was BDH AnalaR grade and glutathione was purchased from Sigma biochemicals. All other chemicals were purchased from BDH chemicals.

Electronic spectra were recorded using a Perkin-Elmer 330 spectrometer and EPR spectra with a Bruker ERD/2000/10 instrument. In the kinetic experiments solutions of glutathione and potassium dichromate were adjusted to pH 7.0 with concentrated sodium hydroxide, preequilibrated, mixed and then introduced into the spectrometer as quickly as possible. We initially observed that the kinetics of the reaction were not affected by saturating all solutions with nitrogen, and subsequently no special precautions to exclude molecular oxygen were taken. Rate constants for the formation and disappearance of chromium(V) were obtained by directly fitting A_{obs} versus time to the integral rate equations reported by Moore *et al.* [22]. A computer programme, utilizing the Marquard alogarith, running on an Archimedes 310 computer, written by Dr P. A. Hamilton of this Department was used.

*Calculation by P. O'Brien on data in ref. 26.

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