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Review

Mechanistic studies of relevance to the biological activities of chromium

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Dedicated to Henry Taube whose insightful mentoring of PAL in reaction kinetics and thermodynamics as a postdoctoral fellow has been a lasting influence

Contents

Abstract

Cellular uptake of Cr(VI), followed by its reduction to Cr(III) with the formation of reactive Cr(V/IV) intermediates, is a generally accepted cause of Cr(VI)-induced genotoxicity and carcinogenicity. Recently, Cr(III) oxidation to Cr(V) and/or Cr(VI) in biological systems came into consideration as a possible reason of anti-diabetic activities of some Cr(III) complexes, as well as of long-term toxicities of such complexes. The current review (with 142 references) summarizes the existing knowledge on the mechanisms of biologically relevant redox reactions of Cr(VI/V/IV/III) complexes. Applications of global kinetic analysis techniques, in conjunction with the methods for characterization of reactive intermediates (such as X-ray absorption spectroscopy, electrospray mass spectrometry, and EPR spectroscopy), for understanding the chemical basis of Cr biological activities, are illustrated with the examples from the authors' research.

Keywords: Chromium; Carcinogenesis; Diabetes; Redox reactions; Kinetics; Mechanism; Global kinetic analysis

1. Introduction

Biological roles of Cr are surrounded by controversy [1]. Currently, Cr(VI) compounds are among 88 recognized (class I) human carcinogens (only 33 of those are discrete chemicals or groups of chemicals) [2]. By contrast, most nutritionists regard Cr(III) as an essential micronutrient, acting as an insulin activator [3,4], although this opinion has been disputed [5]. No Cr(III)-dependent biomolecules, such as enzymes or cofactors, have been unambiguously described as yet [1].

The current understanding of Cr(VI)-induced genotoxicity (reviewed in 2001 and 2003) [1,6] is based on Wetterhahn's uptake-reduction model [7]. Key elements of this model are: (i) the active transport of Cr(VI) into cells (through anion channels for soluble chromates or through phagocytosis for insoluble compounds such as $PbCrO_4$); (ii) the intracellular reduction of Cr(VI) with the formation of potentially DNA-damaging Cr(V/IV) intermediates (which can be stabilized by intracellular ligands) and organic rad-

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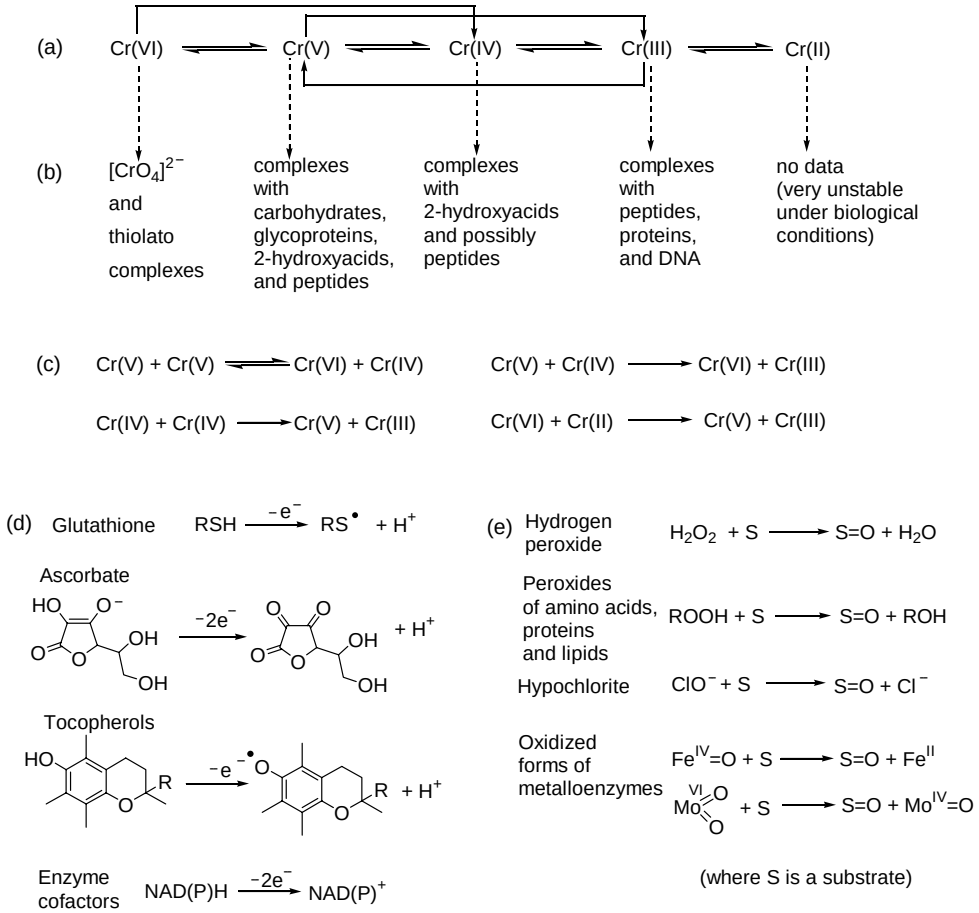
icals; (iii) the formation of kinetically inert Cr(III) complexes, including highly genotoxic DNA–Cr(III)–protein and DNA–Cr(III)–DNA cross-links, as a result of such reduction [7]. Recently, the formation of relatively stable glycoprotein-bound Cr(V) species as a result of Cr(VI) reduction on the cell surface or in extracellular fluids lining the respiratory tract has been added to the above model [8].

There have been fewer studies on the possible involvement of Cr(III) in intracellular redox reactions [6]. The first proposed mechanism [9] involved the reduction of some Cr(III) complexes (including a popular nutritional supplement, Cr(III) picolinate) [10] by ascorbate to Cr(II) species, which then react with O_2, generating reactive oxygen species (ROS). Despite the early claims to the contrary [11], it is now experimentally established [12] that Cr(III) complexes can be oxidized to Cr(VI) in neutral aqueous solutions at ambient conditions by H_2O_2 (which is formed naturally in intracellular processes including redox signalling and immune responses) [13], or by H_2O_2-producing enzymatic systems [12]. Oxidations of certain Cr(III) complexes can lead to stabilization of genotoxic Cr(V) intermediates [14–17]. The Cr(III) + H_2O_2 systems cause extensive oxidative damage to DNA and proteins [18,19], but the nature of the reactive intermediates is unknown. The suggested role of $^{\bullet}$OH radicals as the ultimate DNA-damaging species in such reactions [18] has been disputed, and Cr(V/IV) complexes with peroxo or superoxo ligands have been proposed as possible reactive intermediates [1].

Previously, cellular re-oxidation of Cr(III) was proposed as a possible mechanism of long-term toxicity following exposure to Cr(VI) (which is rapidly reduced to Cr(III) within cells) [6]. It is also possible, however, that the anti-diabetic activities of Cr(III)-containing nutritional supplements [4] involve the formation of Cr(VI), which acts similarly to a well-known [20] insulin mimetic, V(V) (since Cr(VI) and V(V) are isoelectronic). Indeed, experimental observations on insulin-mimetic actions of Cr(VI) have been reported [21–24]. Thus, redox reactions of Cr may provide a link between Cr(VI)-induced genotoxicity and the proposed role of Cr(III) as an activator of insulin-dependent metabolic processes [12]. Such reactions can involve all oxidation states from Cr(VI) to Cr(II), and are complicated by ligand-exchange processes, reactions between Cr species (disproportionation and comproportionation), and formation of organic radicals (capable of initiating radical chain reactions with O_2) [25], as outlined in Scheme 1. Owing to this complexity, the mechanisms of Cr reactions



Scheme 1. An overview of Cr redox chemistry in biological systems: proposed redox transformations of Cr (a); relatively stable complexes of various oxidation states (b); likely reactions between Cr species (c); main reductants (d); and main oxidants (e) [1,6].

under biologically relevant conditions are generally poorly understood [6].

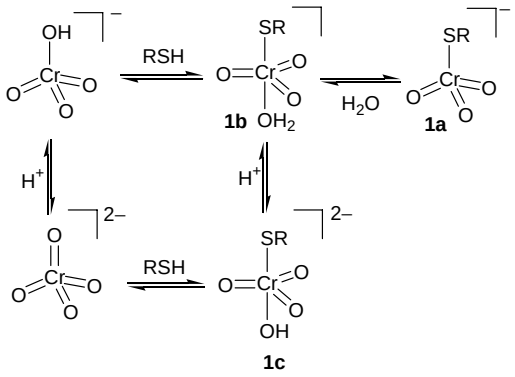
Important developments in the mechanistic studies of Cr reactions in the last decade include: (i) the use of global kinetic analysis (GKA) [6]; (ii) applications of electrospray mass spectrometry (ESMS), EPR spectroscopy and X-ray absorption spectroscopy (XAS) for structural characterization of reactive intermediates [1,26]; and (iii) the development of techniques (based on kinetic analyses) for isolation or quantitative generation of such intermediates [27,28]. The first method, based on kinetic analyses of 3D data sets (absorbance–wavelength–time), was designed primarily for enzymological applications [29–31]. Probably the best known application of GKA to the studies of inorganic reaction mechanisms is related to Ru complexes as redox catalysts [32,33]. The latest developments in GKA involve the use of 4D data sets (with the fourth coordinate being the reaction temperature, reagent concentration, or the pH value) [34–36].

The current review aims to: (i) provide an overview of the existing mechanistic knowledge on the reactions of Cr complexes with biomolecules (see also [6] for a comprehensive survey of the studies published before 2001); and (ii) show some representative examples (mostly from our own work) of the use of kinetic analyses, in conjunction with other techniques, in the mechanistic studies of Cr redox reactions. The approach that we have adopted is to dissect the complex redox chemistry into small components, and to study all steps in detail. A detailed knowledge on the structures of reactive intermediates in various oxidation states of Cr is obtained by isolating such intermediates or generating them in a pure form in solutions. This knowledge, combined with kinetic analyses of the redox reactions, can provide the detailed mechanistic information required to gain further insights into bioinorganic chemistry of Cr. Such approaches should find more general applications in the studies of redox reactions of transition metal complexes.

2. Mechanisms of Cr(VI) Reductions

2.1. Cr(VI) Intermediates

Formation of a Cr(VI)–substrate complex is generally considered as a first step of Cr(VI) reduction reactions [6]. By far the most studied species of this type are Cr(VI) thioesters, which can be easily detected by UV-Vis spectroscopy ($\lambda_{max} \sim 430$ nm, $\varepsilon_{max} \sim 1.2 \times 10^3\ M^{-1}\,cm^{-1}$) during the reactions of Cr(VI) with biological thiols (RSH in Scheme 2), particularly at pH $\leq$ 6 ([6], and references therein). A tetrahedral structure **1a** (Scheme 2) has been proposed for such intermediates, based on multinuclear (^{1}H, ^{13}C, and ^{17}O) NMR spectroscopic studies [37,38]. The structure of a model complex, $(Ph_4As)[CrO_3(SR)]$ (where RSH = 4-bromobenzenethiol), corresponding to **1a**, has been determined by X-ray crystallography [39]. More



Scheme 2. Proposed equilibria involving Cr(VI) oxo and thiolato complexes in aqueous solutions (adapted from [28]).

recent studies by XAS, ESMS, and UV-Vis spectroscopy have shown that Cr(VI) thiolato complexes retain structure **1a** in the solid state, in the gas phase (under ESMS conditions), or in polar aprotic solvents, while five-coordinate species **1b** (Scheme 2) are formed reversibly in aqueous solutions [28]. This is the first observation of a reversible ligand addition reaction in Cr(VI) complexes (such reactions, unlike for ligand substitution, are believed to be uncommon in coordination chemistry) [40]. For instance, a Cr(VI) complex of the most abundant biological reductant, glutathione (GSH, γ-Glu–Cys–Gly) [41] was quantitatively generated in aqueous solutions (pH $\sim$ 3), rapidly frozen to $\sim$10 K for XAS studies, and its structure (corresponding to **1b**) was determined by analysis of its X-ray absorption fine structure (XAFS) [28].

No direct spectroscopic evidence has been obtained, as yet, for the formation of Cr(VI) complexes with biological reductants other than thiols in neutral aqueous solutions, although the formation of a transient Cr(VI)-ascorbato intermediate has been suggested from kinetic data [42–44]. Moreover, several Cr(VI) complexes with non-biological *O*-donor ligands (of a general structure $[CrO_2(OR)_2]$) have been characterized by X-ray crystallography ([45], and references therein). Finally, the formation of Cr(VI) oxalato complexes during the reduction of Cr(VI) with oxalic acid in acidic aqueous solutions has been detected by ^{13}C NMR spectroscopy [46].

Application of the GKA technique has allowed the calculation of UV-Vis spectra for Cr(VI)-thiolato intermediates in neutral aqueous solutions, where such complexes are formed as minor species in mixtures with $[CrO_4]^{2-}$, Cr(V), and Cr(III) [28,47]. An example of GKA for Cr(VI) reduction by GSH is given in Fig. 1 (based on data from [28]). In a typical kinetic experiment [28], 250 time-dependent spectra (in the 330–740 nm range) were collected within 1000 s, using a stopped-flow spectrophotometer with a diode-array detector. A logarithmic time base was used (i.e., time intervals between the spectral measurements increased from 0.03 to 50 s as the reaction progressed), which has allowed a detailed study of the initial fast stages of the reaction. Formation of

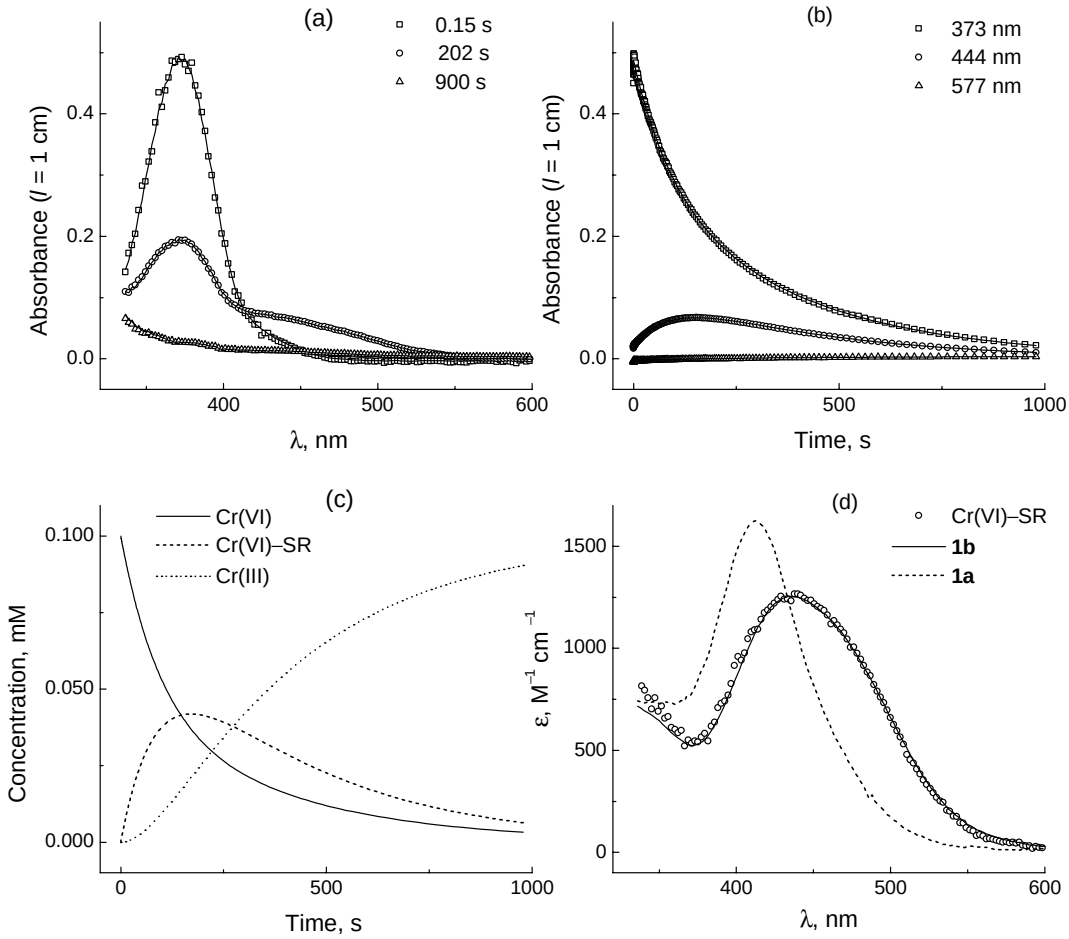
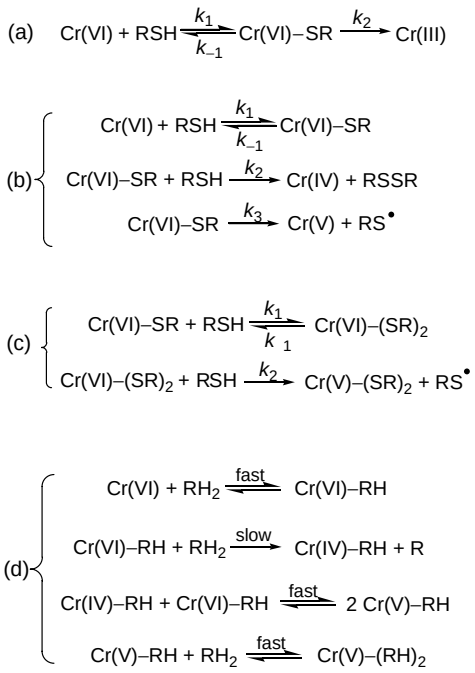


Fig. 1. Typical results of GKA for the reaction of Cr(VI) (0.10 mM) with GSH (20 mM) in HEPES buffer (HEPES = *N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid], 0.10 M, pH = 7.4) at 22 °C in Ar-saturated solution (based on data from [28]). The applied kinetic scheme corresponded to Scheme 3a, optimized rate constants: $k_1 = 7.7 \times 10^{-3}\,s^{-1}$, $k_{-1} = 2.7 \times 10^{-3}\,s^{-1}$, and $k_2 = 4.0 \times 10^{-3}\,s^{-1}$. Presented data: (a) characteristic experimental (symbols) and fitted (lines) spectra at different time points; (b) characteristic experimental (symbols) and fitted (lines) kinetic curves at different wavelengths; (c) calculated kinetic curves for the reacting species; and (d) calculated spectrum of the Cr(VI)−SR intermediate in comparison with that of '1a' (in *N*,*N*-dimethylformamide solution) and '1b' (in aqueous solution, pH = 3.0).

an intermediate, that absorbs at 400–500 nm, was evident from the inspection of separate spectra and kinetic curves (Fig. 1a and b), and was confirmed by mathematical analysis of the whole data set (using the singular value decomposition procedure, which is independent of kinetic modelling) [29–31].

The proposed kinetic scheme (Scheme 3a) was identical to that used previously for the studies of the same reaction by fitting of single kinetic curves (at 425 nm) [48]. However, the use of GKA provided extra confidence in the results of kinetic analyses, since an excellent fit of the experimental data by Scheme 3a was achieved at all the wavelengths and reaction times (typical examples are shown in Fig. 1a and b). The formation of a Cr(VI) thiolato complex as the only detectable intermediate under the studied conditions (Fig. 1) was confirmed by ESMS and EPR spectroscopy (which did not detect any Cr(V) species, see Section 2.2) [28]. The maximal concentration of the Cr(VI)–SR intermediate (Scheme 3a) reached ∼40% of total Cr (Fig. 1c), and its calculated spectrum was independent of the reaction conditions and corresponded to that of **1b** (not **1a**, Fig. 1d and Scheme 2) [28]. A significant decrease in stability of **1b** in neutral versus acidic aqueous solutions can be explained by the set of equilibria in Scheme 2, where **1c** is an unstable intermediate that is not observed spectroscopically [28]. The consequences of the formation of **1b** (not **1a**, as thought before) [37,38] in aqueous solutions for the reactivity of Cr(VI) in cellular media are yet to be explored. One possible implication involves the reactions of Cr(VI) with Cys residues in the active sites of enzymes (Section 4).

Intramolecular electron transfer within Cr(VI)–substrate complexes is a key step of Cr(VI) reductions, and is responsible for the formation of potentially genotoxic Cr(V), Cr(IV), and radical intermediates [6]. Possible mechanisms of this step are still under dispute, even for the much studied reactions of Cr(VI) with thiols in neutral aqueous solutions. Most authors [49–51] proposed a combination of two- and one-electron steps, leading to Cr(IV) and Cr(V), respectively



Scheme 3. (a–d) Selected mechanistic schemes used in the kinetic modelling of Cr(VI) reductions.

(Scheme 3b), based on single-wavelength (~370 nm) kinetic studies of Cr(VI) reductions by thiols. It was assumed that the two-electron pathway becomes dominant when a thiol is present in a large excess with respect to Cr(VI), and the reaction kinetics then corresponds to Eq. (1) [50,52]:

$$k_{obs} = \frac{a[RSH]^2}{1 + b[RSH]} \quad (1)$$

where $a = k_2k_1/k_{-1}$ (Scheme 3b), and $b = k_2/k_{-1}$ (when Cr(VI)−SR is a steady-state intermediate), or $b = k_1/k_{-1}$ (when Cr(VI)–SR is formed in a fast pre-equilibrium step) [49,53]. There are two main limitations to this approach. First, following the reactions at ~370 nm provided very limited information on the formation and decomposition kinetics of the Cr(VI)–SR intermediates ($\lambda_{max} \sim 430$ nm) [47]. Second, the reactions in Scheme 3b assume the formation of aquated Cr(V/IV) ions (rather than Cr(V/IV)–substrate complexes), which is unreasonable for neutral aqueous media [6]. Studies of the Cr(VI) reduction by L-cysteine (Cys), using the GKA approach, allowed for a clear separation of the formation and decomposition steps for the Cr(VI)–SR intermediate, and showed that Eq. (1) applies to the decay of this intermediate, rather than that of total Cr(VI) [47]. This finding led to a proposed mechanism (Scheme 3c), in which a relatively stable Cr(VI) thiolato complex (probably of the structure **1b**, Scheme 2) [28] initially forms an unstable Cr(VI) bis-thiolato intermediate (not observed spectroscopically), which then reacts with another RSH molecule, leading to a bis-ligated Cr(V) thiolato complex. The latter is consistent with the current knowledge on the structures of Cr(V) complexes (including those of thiolato ligands, Section 2.2) formed in neutral aqueous solutions [1,6,45].

The above example clearly shows that mechanistic interpretations of Cr(VI) reduction kinetics are dependent on a knowledge of the structures and formation mechanisms of Cr(V/IV) intermediates. Unfortunately, concentrations of such intermediates in biologically relevant reactions of Cr(VI) are generally too low to be followed by GKA of UV-Vis spectral data [47]. One exception is a reaction of Cr(VI) with GSH at high substrate concentrations (≥0.1 M, i.e., in non-biological range) [41], where the formation and decay of Cr(V) intermediate(s) can be observed at $\lambda_{max} \sim 630$ nm (Section 2.2) [54]. Alternative techniques used for the studies of Cr(V/IV) intermediates are described in Sections 2.2 and 2.3.

2.2. Cr(V) Intermediates

The roles of reactive Cr(V) intermediates in DNA damage (Section 4) are currently the focus of studies of chemical mechanisms involved in Cr(VI) toxicity ([1,6,55], and references therein). These studies are greatly facilitated by the use of EPR spectroscopy for selective detection and identification of micromolar concentrations of monomeric Cr(V) species (d^1 systems) in solutions at room temperature [1]. The first observation in the early 1980's of the formation of Cr(V) intermediates during the reduction of Cr(VI) by extracts of mammalian tissues [56] provided a strong impetus for the development of the uptake-reduction model of Cr(VI)-induced carcinogenicity [7]. Since then, an enormous amount of experimental data has been acquired on the formation of Cr(V) intermediates during the reactions of Cr(VI) with biological reductants in vitro, as well as in live animals and plants (reviewed in [1,6,8,57,58]). The main focus of these studies was on structural and thermodynamic aspects of Cr(V) chemistry, rather than on kinetics and mechanisms of redox reactions involving Cr(V) species. Recently, a combination of XAS, ESMS, and EPR and UV-Vis spectroscopic techniques has been applied for the first definitive structural characterization of a Cr(V)–GSH complex **2** (Plate 1) [27], isolated from the reaction of Cr(VI) with GSH at high reagent concentrations [59].

Probably the most important biological implication of Cr(V) chemistry, revealed by EPR spectroscopy, is the stabilization of Cr(V) by 1,2-diolato moieties of carbohydrates and glycoproteins in neutral aqueous solutions [8]. Since carbohydrates reduce Cr(VI) to a significant extent only in strongly acidic media [60], it is likely that the first Cr(V) complexes formed in biological systems upon the addition of Cr(VI) are those of strong reductants, such as GSH or ascorbate ([1,6], and references therein). These complexes then undergo ligand-exchange reactions with carbohydrate 1,2-diols, with the formation of thermodynamically more stable Cr(V) species that can persist for days under biological conditions [61]. In weakly acidic media (pH = 4–5), which exist in some cellular com-

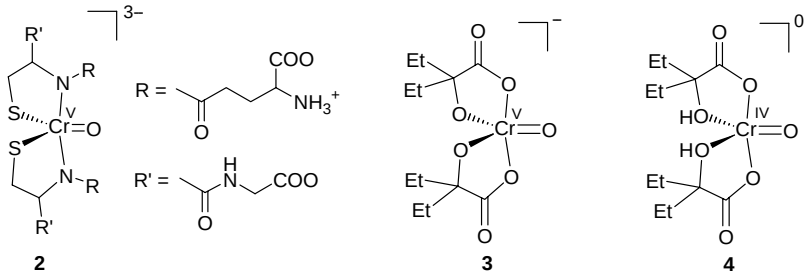


Plate 1. Typical structures of biologically relevant Cr(V/IV) complexes.

partments (e.g., lysosomes), Cr(V) is more likely to be stabilized by 2-hydroxycarboxylato ligands such as citrate [62,63]. Mixed-ligand Cr(V)–substrate-peroxo complexes are formed during the reactions of Cr(VI) with ascorbate or catecholamines in the presence of aerial O_2 (as a result of H_2O_2 formation by substrate autoxidation) [64–66]; such complexes have been implicated as the likely DNA-damaging species in the Cr(VI) + reductant + O_2 systems [1,65,67].

Applications of EPR spectroscopy to the kinetic analysis and modelling of Cr(V) formation in biological systems are illustrated in Fig. 2. Addition of Cr(VI) (0.50 mM) to A549 cells (a human lung epithelial cell line), suspended in HEPES-buffered saline (HEPES = *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid], pH 7.4, ~10^7 cells in 0.25 ml), led to the appearance of EPR signals at g_{iso} = 1.9858 and 1.9797 (signals 1 and 2, respectively, in Fig. 2a); the relative intensity of the latter signal increased with time. These patterns, including the time-dependent changes in relative intensities of the signals (Fig. 2b), were closely matched (Fig. 2a and b) by using the reaction of Cr(VI) (0.50 mM) with a model system containing GSH (2.0 mM) as a reductant, *cis*-1,2-cyclohexanediol (15 mM) as a complexant (mimicking the diolato moieties of carbohydrates and glycoproteins) [6,8], and bovine serum albumin (5.0 mg ml^{-1}) as a protein background (a protein-rich environment leads to broadening of the EPR signals) in HEPES-buffered saline. The g_{iso} = 1.9797 signal is characteristic for the $[Cr^{V}O(L)_2]^-$ complexes (where LH_2 = 1,2-*diol*) [1,6,8], and the g_{iso} = 1.9858 signal has been assigned to the $[Cr^{V}(O)_2(SR)_2(OH_2)]^-$ species (where RSH = thiol) on the basis of comparative EPR-spectroscopic and ESMS studies of Cr(V) complexes with GSH and related thiols [68]. The latter Cr(V) species are also known to form during the decomposition of **2** in aqueous solutions [27]. Since the concentration of GSH used in the model system (Fig. 2) was within the biological range (1–10 mM) [41], it is likely that GSH is the main reductant of Cr(VI) in A549 cells. Previously, EPR spectra similar to those in Fig. 2a were observed for the reactions of Cr(VI) with A549 cells [69] or with rat erythrocytes [70], although the structures of the Cr(V) species were not assigned. Only the g_{iso} = 1.9797 species were observed in live mice, injected with Cr(VI) [71].

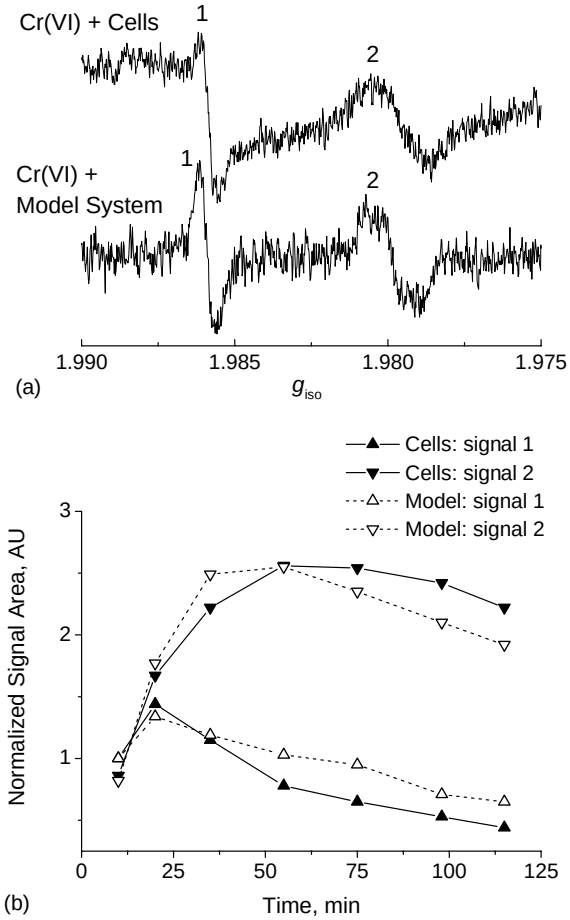
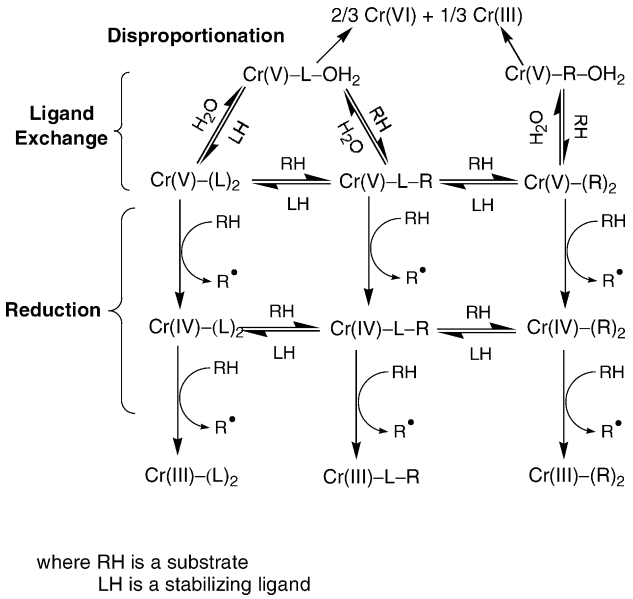


Fig. 2. Typical results of X-band EPR spectroscopy (22 °C) for the reactions of Cr(VI) with A549 (human lung epithelial) cells or with a model system: (a) spectra at 16–24 min after the addition of Cr(VI); and (b) time-dependent changes in relative areas of the signals 1 and 2 (for each experimental series, the area of the signal 1 in the first experiment was taken as a reference). Averages of 20 scans (total scan time 8 min) were used for the data processing; time points in Fig. 1b correspond to the middle of each scan. For the cell system, A549 cells (~10^7) were suspended in reaction solution (0.25 ml); ~90% of the cells retained their membrane integrity after the EPR experiment (determined by trypan blue exclusion). The model system consisted of: GSH (2.0 mM), *cis*-1,2-cyclohexanediol (15 mM), and bovine serum albumin (5.0 mg ml^{-1}). For the both systems, the reactions with Cr(VI) (0.50 mM, Na_2CrO_4) were performed at 22 °C in HEPES-buffered saline (HEPES, 20 mM; NaCl, 140 mM; KCl, 5.0 mM; $MgSO_4$, 2.5 mM; and $CaCl_2$, 1.0 mM; pH = 7.4). The EPR-spectroscopic and cell culture techniques corresponded to those described in [27] and [69], respectively.

Formation of complex **2** (detected by its characteristic EPR signal at $g_{iso} = 1.9955$ or by UV-Vis absorbance at $\lambda_{max} = 630\,nm$, $\varepsilon_{max} = 5.9 \times 102\,M^{-1}\,cm^{-1}$) during the reduction of Cr(VI) by GSH becomes more prominent as the concentration of the reductant increases [27,59]. This seemingly paradoxical observation (as Cr(V) is usually rapidly reduced to Cr(III) by excess reductant) [6] was explained by kinetic studies, revealing the second- and first-order rate equations (with respect to [GSH]) for the formation and decomposition, respectively, of complex **2** [54]. A second-order rate equation (with respect to [RSH]) for the formation of a Cr(V) thiolato complex (such as **2**) is consistent with a sequence of reactions in Scheme 3c, where a thiol acts as a one-electron reductant. By contrast, the highest concentrations of Cr(V) are formed during the reactions of ascorbate or catecholamines (potentially two-electron reductants) with equimolar or slightly higher concentrations of Cr(VI) [43,64,66]. In these cases, reactions of Cr(IV) intermediates with excess Cr(VI) are likely to be responsible for the formation of Cr(V) species (Scheme 3d) [43].

Many aspects of Cr(V) chemistry, including kinetics and mechanisms of its reactions with biomolecules, were studied using a model complex $[Cr^{V}O(ehba)_2]^-$ (**3** in Plate 1), $ehbaH_2$ = 2-ethyl-2-hydroxybutanoic acid) [6,57,72,73], first synthesized by Krumpolc and Roček in the late 1970s [74]. Complex **3** is stable for years in the solid state or months in aprotic organic solvents, but decomposes in aqueous solutions within hours (pH = 3–5) or minutes (in more acidic or basic media) under ambient conditions [75]. This complex is regarded as a model of relatively stable Cr(V) complexes of 2-hydroxycarboxylic acids or 1,2-diols formed in biological systems exposed to Cr(VI) [6]. A simplified mechanism for the reactions of **3** (or similar Cr(V) complexes with biological ligands, designated as Cr(V)–$(L)_2$) in neutral aqueous solutions in the presence or absence of reductants, including GSH, Cys, ascorbate, or Trolox (a water-soluble analog of tocopherols, Scheme 1) is given in Scheme 4 [25,75]. Reduction of Cr(V) is preceded by ligand-exchange reactions with the substrate (Scheme 4), a step that was assumed in earlier studies [57], and more recently directly observed by multi-wavelength stopped-flow UV-Vis spectroscopy [25,47]. Ligand-exchange reactions of **3** with H_2O or a buffer are the first (and often rate-limiting) steps in disproportionation of the complex in neutral aqueous solutions (Scheme 4) [75]. A detailed kinetic study [75] revealed a very complex mechanism of Cr(V) disproportionation under these conditions, involving the formation of several Cr(V) and Cr(IV) intermediates (not included in Scheme 4). Formation of Cr(IV) intermediates (stabilized by ehba ligands) during the reduction of Cr(V) to Cr(III) has been observed even for potentially two-electron reductants such as ascorbate [25]. Methods for observation of Cr(IV) intermediates are described in Section 2.3.



Scheme 4. General mechanism of the reactions of stabilized Cr(V) complexes with biological reductants in neutral aqueous solutions (based on the data from [25,75]). The Cr(V) and Cr(IV) species are five- or six-coordinate with at least one oxo ligand and the Cr(III) complexes are octahedral.

2.3. Cr(IV) Intermediates

Compared with Cr(V) complexes, Cr(IV) intermediates formed in biological reductions of Cr(VI) are much more difficult to study, owing to the short lifetimes of such species and the absence of selective methods for their detection ([6], and references therein). Some of the proposed methods, such as trapping of Cr(IV) by Mn(II) [43] or dynamic magnetic susceptibility measurements in solutions [76] have subsequently been proven invalid for at least some types of Cr(IV) complexes [6,28].

A method for quantitative generation of relatively stable Cr(IV) 2-hydroxycarboxylato complexes (**4** in Plate 1, and its analogues) by the reaction of Cr(VI) with As(III) in weakly acidic (pH = 2–4) aqueous solutions in the presence of excess ligand, developed by Gould and co-workers [77], allowed for the systematic studies of aqueous Cr(IV) chemistry (reviewed in [78]). These studies depend upon monitoring the strong absorbances of **4** and related species in the visible region (400–600 nm) [6,78]. The structure of complex **4** (in frozen aqueous solutions, 10 K) has been determined by XAFS spectroscopy [79,80]. The reported values of formal redox potentials involving **3** and **4** differ from 0.44 to 1.29 V (Cr(V/IV) couple) and from 1.24 to 1.35 V (Cr(IV/III) couple); these discrepancies probably originate in different interpretations of ligand-exchange reactions of **3** and **4** in solutions [81–83]. Application of GKA allowed the calculation of UV-Vis spectra and the assignment of the structures of the products of ligand-exchange reactions of **4**, which have lifetimes of seconds at ambient conditions [25,75,84]. Deprotonation of **4**, leading to

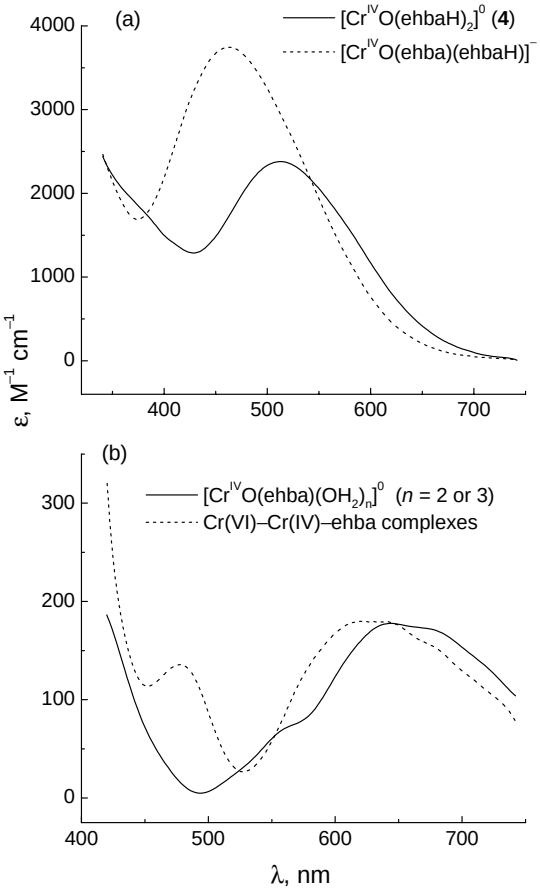


Fig. 3. UV-Vis spectra of bis-ligated (a) and mono-ligated (b) Cr(IV)–ehba complexes in aqueous solutions ([Cr] = 0.10 mM, 25 °C), determined by multi-wavelength stopped-flow spectroscopy (based on the data from [25,75,84]).

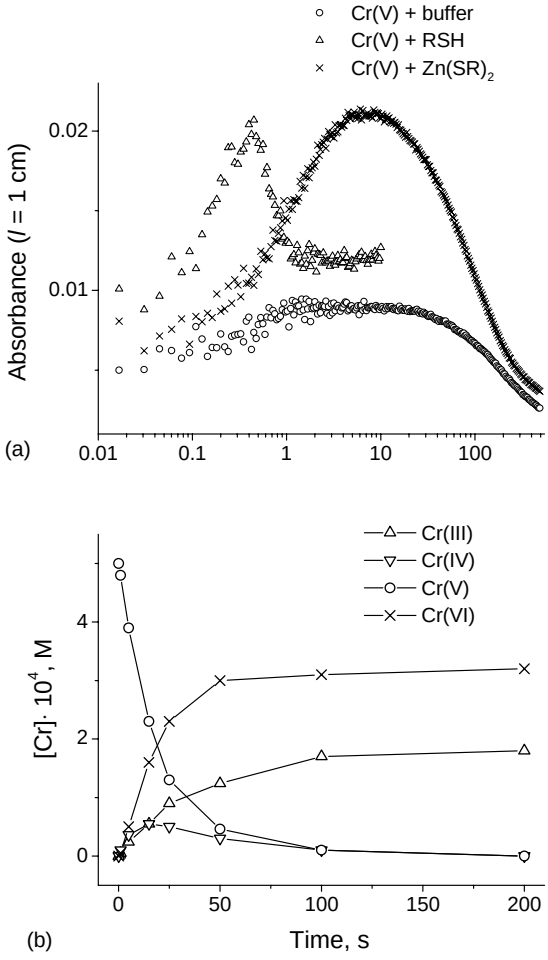


Fig. 4. Formation of Cr(IV) intermediates during the reactions of **3** (0.10 mM) in neutral aqueous solutions (0.10 M HEPES buffer, Ar-saturated, pH 7.4) at 25 °C: (a) kinetic curves at 624 nm for the reactions with *O*-ethyl-L-cysteine (RSH, 4.0 mM), its Zn(II) complex (2.0 mM Zn), or the buffer alone (based on the data from [88]); (b) changes in the concentrations of different oxidation states of Cr during the reaction of **3** with the buffer (determined by quenching studies, based on the data from [75]).

$[Cr^{IV}O(ehba)(ehbaH)]^-$ (pK_a = 5.2), is manifested by a shift in the λ_{max} value from 520 to 460 nm, as well as in an increase in the ε_{max} value (Fig. 3a) [25,75]. A partial aquation of **4** with the formation of $[Cr^{IV}O(ehba)(OH_2)_n]^0$ (n = 2 or 3) is accompanied by a dramatic decrease of absorbance in the visible region and with a shift in the λ_{max} value to ~640 nm (Fig. 3a and b) [75,84].

Other chelating carboxylato ligands, including oxalate and picolinate, are also capable of stabilizing Cr(IV) in aqueous solutions (pH = 2–6) [6,84]. Importantly, Cr(IV) is not stabilized by carbohydrate ligands (unlike Cr(V)) [6,84]. Recently, a stable Cr(IV) complex with a synthetic tripeptide ligand has been quantitatively generated by bulk electrolysis of a corresponding Cr(V) complex (in non-aqueous solutions) and structurally characterized by XAFS spectroscopy [26,85]. Complexes of Cr(IV) with peptide or protein ligands may be involved in the formation of genotoxic protein–Cr(III)–DNA cross-links [1], but experimental evidence for such a role has yet to be obtained.

Formation of Cr(IV) during the reactions of Cr(VI) with two-electron reductants in weakly acidic aqueous media (pH = 2–4) or with aliphatic alcohols (which act as both reductants and solvents) can easily be detected from the development of a red color (characteristic of **4**) when $ehbaH_2$ is present in the reaction mixture in a large excess (typically, ≥0.1 M at [Cr] ≤1 mM) [78,86,87]. However, these conditions have only limited implications to biological systems. Intermediate absorbances with λ_{max} ~ 600–650 nm (due to the Cr(IV)–ehba–H_2O and/or Cr(IV)–ehba–substrate complexes, Fig. 3b) were observed during the disproportionation of **3**, as well as in the reactions of **3** with several major biological reductants or their models (all reactions were carried out at pH = 7.4 in the absence of added ehba ligand) [25,75]. Shown in Fig. 4a are typical kinetic curves at 624 nm for the reactions of **3** with a stable tetrahedral complex, $[Zn(SR)_2]$ (where RSH = *O*-ethyl-L-cysteine), used as a model of a Zn(II) center in zinc finger proteins (Section 4), as well as with the free ligand (RSH) [88]. Thus, direct observation of Cr(IV) intermediates of Cr(V) reductions under biologically

relevant conditions is possible (using multi-wavelength stopped-flow spectroscopy), but their quantitation is difficult, since the absorbance of the small amounts of Cr(IV) thus generated is comparable in intensity to those of the predominant Cr(V) and Cr(III) species [25]. Furthermore, this method cannot be applied to the detection of Cr(IV) intermediates formed during the reduction of Cr(VI), due to very low steady-state concentrations of such intermediates [47]. Application of ESMS for the detection of Cr(IV) intermediates in such systems is also difficult due to the presence of large excesses of other ionic species and secondary redox reactions under ESMS conditions, or due to the formation of uncharged Cr(IV) complexes, such as **4** [88].

To quantify the formed Cr(IV) intermediates, reactions of **3** with a buffer or a reductant were allowed to proceed for a certain time, and then the reaction solution was mixed with a concentrated solution of $ehbaH_2$/$ehbaH^-$ buffer (pH = 3.0), using a sequential mixing facility of a SX-17MV stopped-flow spectrophotometer (Applied Photophysics, UK) [75]. These conditions lead to quenching of all the transient Cr(IV) species into a relatively stable complex, **4**, and allow the calculations of concentrations and UV-Vis spectra of these species from the spectra of the reaction mixture before and after the quenching step (since the spectra of Cr(VI), Cr(V), and Cr(III) species are known) [75]. Detailed quenching studies, performed for the disproportionation of **3** in neutral aqueous media [75], provided the first direct evidence for the formation of Cr(IV) intermediates under these conditions, which was important for the analysis of mechanisms of DNA damage induced by **3** (Section 4). Calculated spectra of the formed Cr(IV) species were similar, but not identical, to those of $[Cr^{IV}O(ehba)(OH_2)_n]^0$ (n = 2 or 3, Fig. 3b), and were assigned to dimeric Cr(VI)–Cr(IV)–ehba complexes, based on an analysis of the kinetic data [75]. The quenching method can potentially be applied to quantitation and spectral characterization of Cr(V/IV) intermediates formed during the reactions of Cr(VI) with biological reductants. One drawback of this method is that it is time-consuming, since acquisition of each time point (e.g., in Fig. 4b) requires a separate kinetic experiment.

2.4. Cr(III) Intermediates and products

The well-known kinetic inertness of Cr(III) [45,89] provides an additional option for the mechanistic studies of Cr(VI) reductions by biomolecules, since the structures of Cr(III) products are likely to reflect those of kinetically labile Cr(V/IV) precursors [6,47,90]. A major problem in the studies of Cr(III) products of Cr(VI) reductions in neutral aqueous media is a relatively rapid hydrolysis of the initially formed Cr(III) species, leading to polynuclear Cr(III) hydroxo complexes [45]. New insights into the solution chemistry of Cr(III) complexes, formed in Cr(VI) reductions, have been obtained recently with the use of ESMS and XAS (see below).

(a)
$$Cr(VI) + 3\,RSH \xrightarrow{pH < 4} Cr(III) + 3/2\,RSSR$$
$$Cr(VI) + 5\,RSH \xrightarrow{pH > 6} Cr^{III}(SR)_2 + 3/2\,RSSR$$

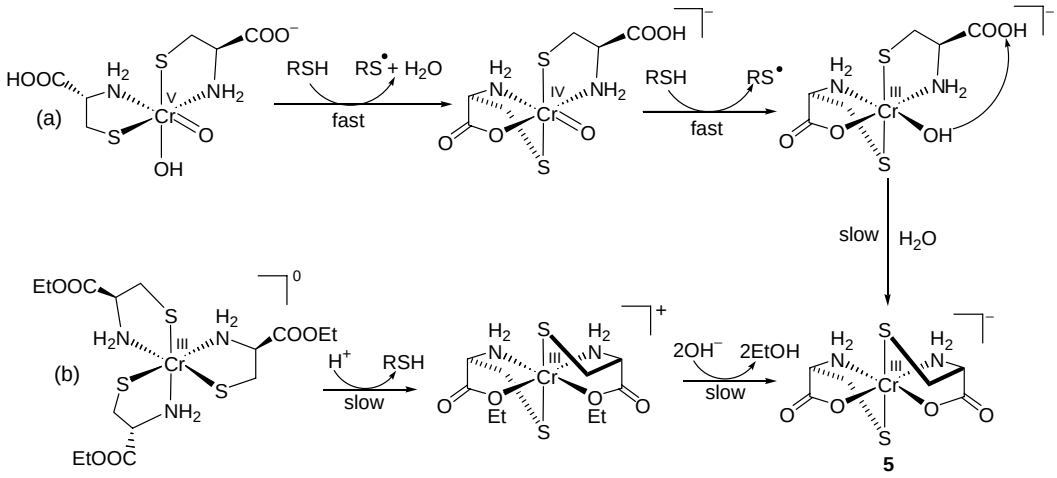
(b)
$$3\,[\underset{\mathbf{3}}{Cr^{V}O(ehba)_2}]^- + 9\,H_2O \xrightarrow{pH = 6\text{-}8} [Cr^{III}(ehba)(OH_2)_4]^+ + 2\,[CrO_4]^{2-} + 5\,ehbaH^- + 5\,H^+$$

Scheme 5. Stoichiometries of Cr(VI) reductions by Cys (RSH) in acidic and neutral aqueous solutions (a), and of the disproportionation of complex **3** in neutral aqueous solutions (b), based on the data from Refs. [47,75,91,92].

In some cases, stoichiometric studies of Cr(VI/V) reductions provided valuable information on the structures of Cr(III) products. For instance, Cys and other thiols act as one-electron reductants towards Cr(VI), so that 3 mol of a thiol are spent for the reduction of 1 mol of Cr(VI) to Cr(III) in acidic media (Scheme 5a) [91]. Under these conditions, Cr(III) is likely to form complexes with aqua and/or buffer ligands [90]. By contrast, 5 mol of Cys are required for the reduction of 1 mol of Cr(VI) in neutral aqueous media, due to the formation of a stable Cr(III) bis-cysteinato complex (Scheme 5a) [47,92]. Formation of $[Cr^{III}(ehba)(OH_2)_4]^+$ during the disproportionation of **3** in neutral aqueous solutions has been deduced from the decreases in pH values of the reaction solutions (5 mol of H^+ are formed per 3 mol of decomposed Cr(V), Scheme 5b) [75]. These cationic Cr(III) species are thought to be responsible for the Cr(III)–DNA binding caused by disproportionation of **3** [93]. In the absence of DNA, $[Cr^{III}(ehba)(OH_2)_4]^+$ undergoes base hydrolysis with the formation of insoluble Cr(III) hydroxo complexes [93].

The $[Cr^{III}(cys)_2]$ complex ($cysH_2$ = Cys), formed during the reduction of Cr(VI) by excess Cys at pH = 6.5–7.5, was identical (on the basis of its UV-Vis and CD spectra) [47,92] to that isolated from the reaction of Cr(III) with Cys (at pH ∼ 8), which was characterized by X-ray crystallography (**5** in Scheme 6) [94]. Hydrolysis of **5** is negligibly slow at the timescale of the Cr(VI) reduction reaction, due to stabilization of the Cr(III) complex by chelating cys ligands [47]. A knowledge of the structure of **5**, together with detailed kinetic analyses (by GKA) of Cr(VI) reduction by excess Cys at pH ∼ 7, allowed for assignment of possible structures of Cr(IV) and Cr(V) intermediates (Scheme 6a) [47]. While these intermediates were not observed experimentally due to their high reactivities, the first direct spectroscopic evidence was obtained for the formation of a precursor Cr(III) complex, which then converts to **5**, probably via internal lactonization [95] (Scheme 6a, proposed based on the independence of the reaction rate constant on the ligand concentration and the pH value) [47].

Three main Cr(III) products were observed by ESMS following the reduction of Cr(VI) by excess *O*-ethyl-L-cysteine in neutral aqueous media (Scheme 6b) [88]. The initially



Scheme 6. Proposed structures of Cr(V/IV/III) intermediates leading to a structurally characterized Cr(III) complex **5** during the reductions of Cr(VI) by Cys (a) or by *O*-ethyl-L-cysteine (b), based on the data from Refs. [47,88].

formed tris-ligated Cr(III) complex is converted to a bis-ligated form, which then undergoes a slow intramolecular hydrolysis with the formation of **5**; the latter reaction is driven by the high affinity of Cr(III) for deprotonated carboxylato donors [88]. Such intramolecular reactions may be important for biological functions of Cr(III) [1].

Reduction of Cr(VI) by GSH in neutral aqueous solutions leads to a mixture of Cr(III) complexes with reduced (GSH) and oxidized (GSSG) forms of the substrate, where Cr(III) most likely binds to amino and carboxylato donors of the glutamine residue (as shown by ESMS and XAS studies) [27]. These findings are in marked contrast with the structure of a relatively stable Cr(V)–GSH intermediate (**2**), where the Cr(V) center is bound to the thiolato and deprotonated amido donors of the Cys residue [27]. Such a difference in ligation types implies the formation of several reactive Cr(V/IV) species as intermediates between **2** and the Cr(III) products, but the mechanisms for the formation of such intermediates are still unclear [27]. Many Cr(III) complexes have been isolated from the reactions of Cr(VI) with GSH, ascorbate and other biologically relevant ligands in vitro [96–100], but no definitive structural information on such complexes is available as yet, and the relationships of the isolated compounds to initial Cr(III) species (kinetic products) formed in aqueous solutions are uncertain.

In summary, some progress has been made recently in the characterization of Cr(III) products of Cr(VI) reductions with biomolecules (mostly thiols), but much more remains to be learned in this area. Future studies are likely to be directed towards an understanding of the structures and formation mechanisms of (amino acid)–Cr(III)–DNA, protein–Cr(III)–DNA and DNA–Cr(III)–DNA cross-links, which have been hypothesized by many researchers as the main species that ultimately lead to Cr(VI)-induced carcinogenicity ([1], and references therein). Such studies will probably involve detailed kinetic analyses of the reductions of Cr(VI) (e.g., with GSH or ascorbate) in the presence of model peptides and/or oligonucleotides.

2.5. Cr(II) Intermediates

The formation of Cr(II) intermediates during the reactions of Cr(VI) with two-electron reductants (such as alcohols, phenols, or aldehydes) in strongly acidic aqueous media (pH = 0–1) is well established; a likely mechanism is presented by Eqs. (2)–(6), where RH_2 and R are the reduced and oxidized forms of the substrate [101,102]:

$$[HCr^{VI}O_4]^- + RH_2 + 3H^+ \rightarrow Cr^{IV}O^{2+}(aq) + R + 3H_2O \quad (2)$$

$$Cr^{IV}O^{2+}(aq) + RH_2 \rightarrow Cr^{II}(aq) + R + H_2O \quad (3)$$

$$Cr^{II}(aq) + [HCr^{VI}O_4]^- \rightarrow Cr^{V}(aq) + Cr^{III}(aq) \quad (4)$$

$$2Cr^{V}(aq) \rightarrow [HCr^{VI}O_4]^- + Cr^{IV}O^{2+}(aq) \quad (5)$$

$$Cr^{II}(aq) + O_2 \rightarrow Cr^{III}(O_2)^{2+}(aq) \quad (6)$$

Such Cr(II) intermediates are trapped either by the reactions with O_2, leading to Cr(III) peroxo species (Eq. (6)), which possess characteristic absorbances at 250–300 nm or, in the absence of O_2, by the reactions with Co(III) ammine complexes [101,102]. The ability of aquated Cr(IV) to react with two-electron reductants by hydride-transfer mechanism with the formation of Cr(II) (Eq. (3)) has been confirmed independently by generation and detailed reactivity studies of $Cr^{IV}O^{2+}(aq)$ (half-life time ~30 s at pH = 0 and 25 °C) [101,102].

By contrast, there is no firm evidence for the formation of Cr(II) intermediates under biologically relevant conditions, in agreement with the extremely high susceptibility of such species to base hydrolysis and oxidation by O_2 [89]. Appearance of UV-Vis spectra, similar to those of $Cr^{III}(O_2)^{2+}(aq)$ (Eq. (6)), has been reported for the reactions of Cr(VI) with

GSH or related thiols in the presence of O_2 at pH = 5.4 [103], but interpretation of spectral data under these conditions is complicated by the formation of disulfides absorbing in the same region [47]. The possibility of the formation of Cr(III) complexes with O_2-derived ligands during the reduction of Cr(VI) in neutral aerated aqueous solutions warrants further investigation as a potentially important route in Cr(VI)-induced genotoxicity (Section 3). Formation of Cr(II) intermediates and their subsequent reactions with O_2 have been implied as the main cause of oxidative DNA damage and mutagenicity induced by some types of Cr(III) complexes [9,10]. However, alternative explanations of these phenomena, involving the formation of Cr(V/IV) species, are more likely [1].

2.6. Free radical intermediates

Applications of EPR spectroscopy for the detection of free radical species formed in redox reactions of Cr complexes, including the ambiguities in interpretation of the results of spin-trap studies, have been described in detail previously [1]. This section concentrates on the use of GKA and oxygen consumption data for the mechanistic studies of Cr(VI/V/IV) reductions in aerated aqueous media, leading to the formation of sulfur-, carbon-, and oxygen-based radicals [25,88].

Comparative kinetic studies of the reactions of Cr(VI), Cr(V) (**3**), or Cr(IV) (**4**) with major biological reductants in vitro point to a close relationship between the formation of Cr(V/IV) species and organic radicals in biological reductions of Cr(VI) [25]. Direct UV-Vis spectroscopic evidence has been obtained for the formation of *O*-based anion–radicals of ascorbate (λ_{max} = 360 nm) or Trolox (λ_{max} = 435 nm) during the reactions **3** or **4** with the corresponding reductants, using the GKA technique [25]. Formation of *C*-based radicals during the reactions of Cr(V) (but not Cr(IV)) with ascorbate in neutral aqueous solutions is manifested by an unusual reaction stoichiometry, whereby 1.0 mol of Cr(V) is completely reduced to Cr(III) by 0.65 mol of ascorbate (instead of the 1:1 ratio expected for ascorbate as a two-electron reductant, Scheme 1) [25]. A proposed mechanism for the formation of such radicals ($R_{ox}^{\bullet}$) is shown in Scheme 7a [25]. Formation of *C*-based radicals has also been detected during the reaction of Cr(VI) with ascorbate, using EPR spectroscopy and spin trapping techniques [43].

Reactions of Cr(VI/V/IV) with ascorbate, GSH, or Cys in neutral (or weakly acidic) aerated aqueous solutions are accompanied by O_2 consumption, which is conveniently measured using a Clark-type oxygen electrode [25]. Mechanisms of O_2 activation during the reactions of Cr(VI/V/IV) with ascorbate (Scheme 7a) or biological thiols (Scheme 7b) have been proposed, based on the comparison of kinetic data for O_2 consumption with those for Cr(VI/V/IV) reduction (obtained by GKA) [25]. Fig. 5a illustrates the influence of inhibitors on the kinetics of O_2 consumption.

(a)

$$RH_2 \rightleftharpoons RH^- + H^+$$

$$RH^- + Cr^{(n+1)+} \longrightarrow R^{\cdot} + Cr^{n+}$$

$$R^{\cdot} + Cr^{(n+1)} \longrightarrow R + Cr^{n+}$$

$$R^{\cdot} + Cr(V) \rightleftharpoons Cr(V)\text{–}R^{\cdot} \longrightarrow Cr(IV)\text{–}R \longrightarrow Cr(III)\text{–}R^{+} \longrightarrow Cr(III) + R_{ox}^{\cdot}$$

$$R^{\cdot} + O_2 \longrightarrow R + O_2^{\cdot -}$$

$$R_{ox}^{\cdot} + O_2 \longrightarrow R_{ox}OO^{\cdot} \longrightarrow \text{oxidation products}$$

$$2O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2$$

(b)

$$RSH \rightleftharpoons RS^- + H^+$$

$$RS^- + Cr^{(n+1)+} \longrightarrow RS^{\cdot} + Cr^{n+}$$

$$RS^- + RS^{\cdot} \rightleftharpoons RSSR^{\cdot -}$$

$$RSSR^{\cdot -} + Cr^{(n+1)+} \longrightarrow RSSR + Cr^{n+}$$

$$RS^{\cdot} + O_2 \longrightarrow RSOO^{\cdot} \longrightarrow \text{oxidation products}$$

$$RSSR^{\cdot -} + O_2 \longrightarrow RSSR + O_2^{\cdot -}$$

$$RSH + O_2^{\cdot -} + H^+ \longrightarrow RS^{\cdot} + H_2O_2$$

(c)

$$[Zn(SR)_2]^0 + Cr^{(n+1)+} \longrightarrow [Zn(SR)(^{\cdot}SR)]^+ + Cr^{n+}$$

$$[Zn(SR)(^{\cdot}SR)]^+ + 2X^- \longrightarrow ZnX_2 + RSSR^{\cdot -}$$

Scheme 7. Main processes leading to the formation of organic radicals and O_2 consumption during the reactions of Cr(VI/V/IV) with biological reductants in neutral or weakly acidic aqueous solutions (based on the data from Refs. [25,88]). Designations: RH_2 = ascorbic acid (a); RSH = GSH, Cys or *O*-ethyl-L-cysteine (b,c); X^- = buffer anion; and n = 3, 4, or 5.

Dimethyl-1-pyrroline *N*-oxide (DMPO, an efficient scavenger of thiyl ($RS^{\bullet}$) radicals [104]), and catalase (a selective H_2O_2 scavenger [105]) inhibit the initial (fast) and final (slow) stages, respectively, of O_2 consumption caused by the reduction of Cr(V) (**3**) with excess Cys at pH = 7 [25]. Thus, the fast stage of O_2 consumption is likely to be due to the formation of $RS^{\bullet}$ radicals in the reactions of Cr(V/IV) with Cys, whereas the slow stage (taking place after the complete reduction of Cr(V) to Cr(III)) is probably due to the reactions of the formed H_2O_2 with excess Cys (Scheme 7b) [25].

Roles of free radicals in the reactions of Cr(VI/V/IV) with biologically important Zn-S bonds (Section 4) were studied using a stable model $[Zn(SR)_2]$ complex (RSH = *O*-ethyl-L-cysteine) [88]. While a model Cr(V) complex, **3**, is rapidly reduced by $[Zn(SR)_2]$ in neutral aqueous solutions, this reaction is not accompanied by a significant O_2

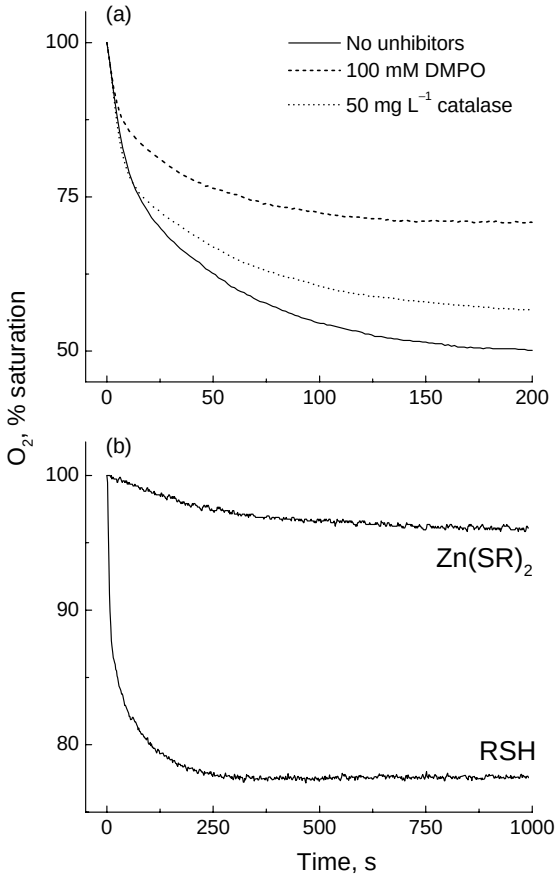


Fig. 5. Typical results of O_2 consumption studies for the reactions of Cr(V) (3) with biological thiols: (a) influence of inhibitors on the reaction of **3** (0.20 mM) with Cys (5.0 mM) in phosphate buffer (0.10 M, pH = 7.0) at 25 °C (based on the data from [25]); and (b) reactions of **3** (0.20 mM) with *O*-ethyl-L-cysteine (RSH, 4.0 mM) or its Zn(II) complex (2.0 mM Zn) in HEPES buffer (0.10 M, pH = 7.4) at 25 °C (based on the data from [88]).

consumption, unlike for the corresponding reaction of the free ligand (RSH, Fig. 5b) [88]. This observation was explained by intramolecular coupling of two thiolato ligands within $[Zn(SR)_2]$, without the release of free RS$^\bullet$ radicals (Scheme 7c) [88]. Similar mechanisms may be involved in regulatory functions of zinc metallothioneins, which are thought to be based on their easy oxidation with the formation of intramolecular S–S bonds within the Zn(II)–Cys clusters, leading to the release of Zn(II) [106].

3. Mechanisms of Cr(III) oxidations

Some likely oxidants of Cr(III) in biological systems are shown in Scheme 1e. Oxidized forms of metalloenzymes (e.g., Fe(IV) centers in cytochromes P450 [107] or Mo(VI) centers in oxotransferases [108]) may carry out Cr(III) oxidations [6,109], but it is not yet known whether any of those enzymes can use Cr(III) complexes as substrates. In many instances, however, Cr(III) complexes were oxidized to Cr(V) and/or Cr(VI) species under mild conditions by non-biological oxo-transfer reagents, such as PbO_2, H_5IO_6 (IO_4^-), or PhIO [14–16]. Non-specific biological oxidants that can react with Cr(III) include H_2O_2, ClO^-, and amino acid, protein or lipid peroxides [1,13]. There are scattered reports in the literature on the oxidations of some types of Cr(III) complexes (with non-biological ligands) to Cr(VI) by H_2O_2 or ClO^- at physiological or near-physiological pH values [110,111], but systematic studies of such reactions for biologically relevant Cr(III) complexes (including the reactions with ROS-producing enzymatic systems) have commenced only very recently [12]. Oxidation of Cr(III) to Cr(VI) by H_2O_2 in strongly alkaline media has been known for decades, and is widely applied in analytical chemistry [112,113].

Typical easily oxidizable Cr(III) complexes (**6–9** in Plate 2) possess the following features: (i) at least two aqua ligands, capable of deprotonation in neutral aqueous media; (ii) electron-rich imine ligands; and (iii) ligands capable of stabilizing Cr(V) intermediates (the second feature does not apply to **8** and **9**) [12,14,15,114,115]. For complexes of the $[Cr^{III}(L–L)_3]^{3+}$ type (where L–L is a chelating ligand), oxidation is preceded by a slow conversion to the $[Cr^{III}(L–L)_2(OH_2)_2]^{3+}$ form [114]. An aqua ligand is proposed to act as a good leaving group for the formation of a Cr(III)–oxidant intermediate (Scheme 8a, usually a rate-determining step of the oxidation reaction) [114,116], while in its deprotonated form the OH^- ligand may catalyze the ligand-exchange step [117,118], or act as a bridging ligand to assist in the inner-sphere electron transfer [119]. Kinetics of Cr(III) oxidation were studied by UV-Vis spectrophotometry (following the accumulation of Cr(VI), λ_{max} = 372 nm), using IO_4^- or *N*-bromosuccinimide as oxidants, and the mechanisms (shown in general form in Scheme 8a) were proposed based on the dependencies of the reaction rates on reagent

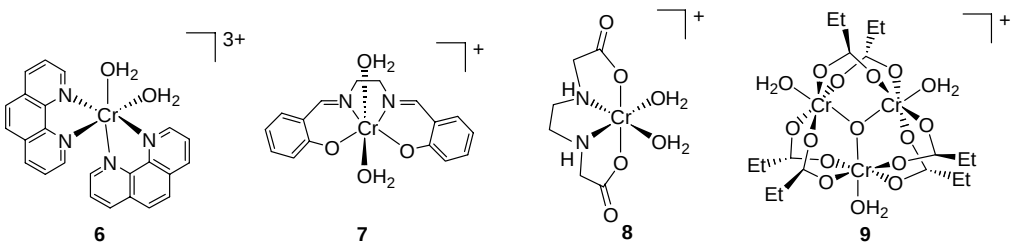
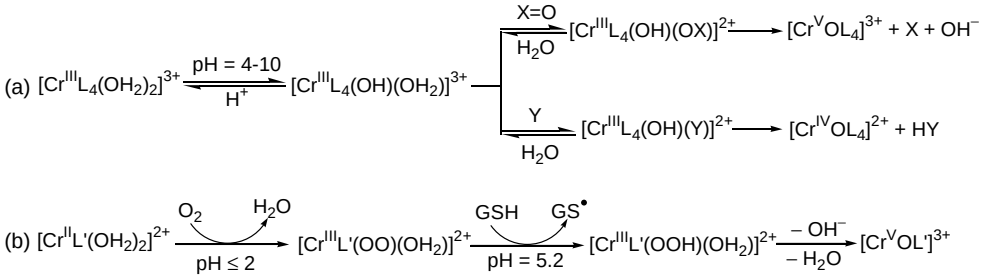


Plate 2. Typical structures of Cr(III) complexes that can be oxidized to Cr(VI/V) species under biologically relevant conditions.



Scheme 8. Proposed mechanisms for the formation of Cr(V/IV) intermediates during Cr(III) oxidations under biologically relevant conditions (based on the data from [114,116,119] for (a) and [122,123] for (b)). Designations: X=O is a two-electron oxidant (*O*-transfer reagent), e.g., IO_4^-; Y is a one-electron oxidant (e.g., *N*-bromosuccinimide); L is a O/N-donor ligand (charges of the complexes can vary dependent on the nature of the ligand); and L′ is 1,4,8,11-tetraazacyclotetradecane (cyclam).

concentrations and the pH values [114,116,119]. There was no direct spectroscopic evidence for the formation of either the intermediate Cr(III)–oxidant complexes or the Cr(V/IV) species (Scheme 8a) [114,116,119]. The latter species are thought to react rapidly with the oxidants, leading to the final Cr(VI) products [114,116,119].

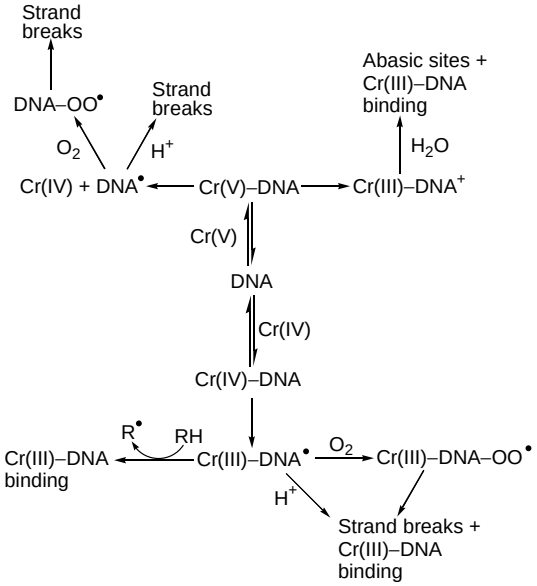
Relatively stable Cr(V) intermediates have been observed by EPR spectroscopy during the oxidations of some types of Cr(III) complexes (such as **6**, **7**, or complexes with oligopeptide ligands) in aqueous solutions [14–16,115]. The following Cr species have been detected by ESMS during the oxidation of **6** (*cis*-$[Cr^{III}(phen)_2(OH_2)_2]^{3+}$, phen = 1,10-phenantroline) by PbO_2 in aqueous acetate buffer (pH = 4.0): $[Cr^{III}(OH)_2(phen)_2]^+$, $[Cr^{V}(O)_2(phen)]^+$, $[Cr^{V}(O)_2(phen)_2]^+$, and $[Cr_2(O)_4(phen)_2]^+$ [115]. Oxidations of **6** and **7** (*trans*-$[Cr^{III}(salen)(OH_2)_2]^+$, salenH$_2$ = *N*,*N*′-ethylenebis(salicylidedeneimine)) with PhIO in non-aqueous media led to the isolations of $[Cr^{V}(O)_2(phen)_2]^+$ (characterized by XAS) [115] and $[Cr^{V}O(salen)]^+$ (characterized by X-ray crystallography) [120]. Isotope (^{18}O) labelling studies showed that the oxo group in $[Cr^{V}O(salen)]^+$ originates from the oxidant, rather than from an aqua ligand in **7** [120]. Stabilities of the Cr(V) oxidation products of **6** and **7** correlate with mutagenicities of these Cr(III) complexes (while most other types of Cr(III) complexes are non-mutagenic) [1,6,14,15]. Recently, a diperoxochromium(V) complex, $[CrL(O_2)_2]$ (where L = hydrotris(3,5-diisopropylryrazolyl)borato(1–)) has been synthesized by oxidation of a Cr(III) precursor, $[CrL(OH)_2(OH_2)]$, with H_2O_2 (in Et_2O solution at 0 °C) [121].

Recent studies by Bakac and co-workers revealed the roles of Cr(III) complexes with O_2-derived ligands in the formation of stabilized Cr(V) species (Scheme 8b) [122,123]. Peroxo complexes such as $[Cr^{III}(cyclam)(OO)(OH_2)]^{2+}$ (Scheme 8b, cyclam = L′ = 1,4,8,11-tetraazacyclotetradecane), formed in the reactions of the corresponding Cr(II) complexes with O_2 in acidic aqueous media, react with one-electron reductants (e.g., GSH or Fe(II)) with the formation of Cr(III) hydroperoxo complexes; the latter undergo an intramolecular redox reaction leading to Cr(V) oxo species such as $[Cr^{V}O(cyclam)]^{3+}$ (Scheme 8b) [122,123]. All the Cr complexes in Scheme 8b were observed by UV-Vis spectroscopy (using a multi-wavelength stopped-flow technique), and the Cr(V) species were also detected by EPR spectroscopy [122,123]. Mechanisms similar to those in Scheme 8b are likely to apply to the oxidations of Cr(II) complexes by organic peroxides in non-aqueous media, leading to stable Cr(V) complexes [85,124]. Oxidations of Cr(III) complexes by H_2O_2 in neutral aqueous media [12,110] may be promoted by the formation of the corresponding Cr(III) hydroperoxo complexes (via ligand exchange) [121], followed by their transformation into Cr(V) oxo complexes (last stage in Scheme 8b), but experimental evidence for such a pathway is yet to be obtained.

4. Mechanisms of DNA and protein damage

Formation of DNA lesions, including strand breaks, abasic sites and Cr(III)–DNA cross-links in Cr(VI)-exposed cells is believed to be the primary cause of Cr(VI)-induced carcinogenicity ([1], and references therein). A detailed recent review on the mechanisms of DNA damage in vitro by Cr complexes in various oxidation states is available [1]. Complexes of Cr(V/IV) with peroxo and/or superoxo ligands have been implicated as likely reactive species that cause oxidative DNA damage on exposure to either the Cr(VI) + reductant + O_2, or Cr(III) + H_2O_2 systems (Cr(VI) itself does not react with isolated DNA) [1,65]. Detailed mechanistic studies of oxidative DNA damage by Cr(V/IV) have been performed with the use of model complexes **3**, **4** (or their analogues), or $[Cr^{V}O(salen)]^+$ [75,125–131]. These studies have unambiguously established the propensity of Cr(V/IV) complexes to react directly with DNA; some of the likely reaction pathways are shown in a general form in Scheme 9 [1].

It is difficult to obtain direct evidence for the roles of reactive intermediates in the oxidative DNA damage caused by the Cr(VI) + reductant + O_2 systems, due to the low steady-state concentrations of such species [132]. Fortunately, some intermediates of Cr(VI) reductions by biomolecules can be either quantitatively generated in



Scheme 9. Main pathways in the oxidative DNA damage by Cr(V/IV) complexes (based on the data from [127]).

acidic aqueous media (**1b**) [28] or isolated in the solid state (**2**) [27]. Conditions for the studies of DNA damage by **1b** or **2** (using a plasmid DNA cleavage assay [127]) were chosen based on the kinetic data for decomposition of these complexes [133]; typical results are illustrated in Fig. 6. Reactions of Cr(VI) ($[CrO_4]^{2-}$) with GSH in neutral aqueous media led to the formation of low concentrations of **1b** (dashed line in Fig. 6a) in equilibrium with $[CrO_4]^{2-}$ (Section 2.1) [28]. Higher concentrations of **1b** in neutral media could be achieved (for ~2 min at 37 °C) when the complex was pre-formed in acidic media (pH = 2.9), and then diluted with a buffer to pH = 7.4 (solid line in Fig. 6a). However, addition of pre-formed **1b** to the buffer led to a smaller amount of DNA cleavage, compared with the reaction of the corresponding amounts of $[CrO_4]^{2-}$ and GSH in the same buffer (conditions 2 and 3 in Fig. 6c). Clearly, **1b** is not directly responsible for the formation of single-strand breaks in plasmid DNA exposed to the Cr(VI) + GSH + O_2 systems [133,134]. Decomposition of **2** in aqueous solutions is practically complete within ~3 min at pH = 7.4 and 37 °C (solid line in Fig. 6b), leading to the formation of Cr(III)–GSH–GSSG complexes (~80% of total Cr) and $[CrO_4]^{2-}$ (dashed and dotted lines in Fig. 6b), due to the parallel disproportionation of Cr(V) and its reduction to Cr(III) by the ligand [27]. In subsequent slow reactions (hours timescale at 37 °C, Fig. 6b), $[CrO_4]^{2-}$ is reduced to Cr(III) by GSH (released during the decomposition of 2) [27]. Fresh solutions of **2** cause much more pronounced DNA cleavage than the same solutions after the decomposition of Cr(V) (conditions 4 versus 5 or 6 in Fig. 6c), although a small amount of DNA cleavage is also observed after the decomposition of **2**, due to the presence of Cr(VI) and GSH (conditions 5 or 6 versus 1 in Fig. 6c). Thus, a Cr(V)–GSH complex, **2**, is capable of inducing oxidative

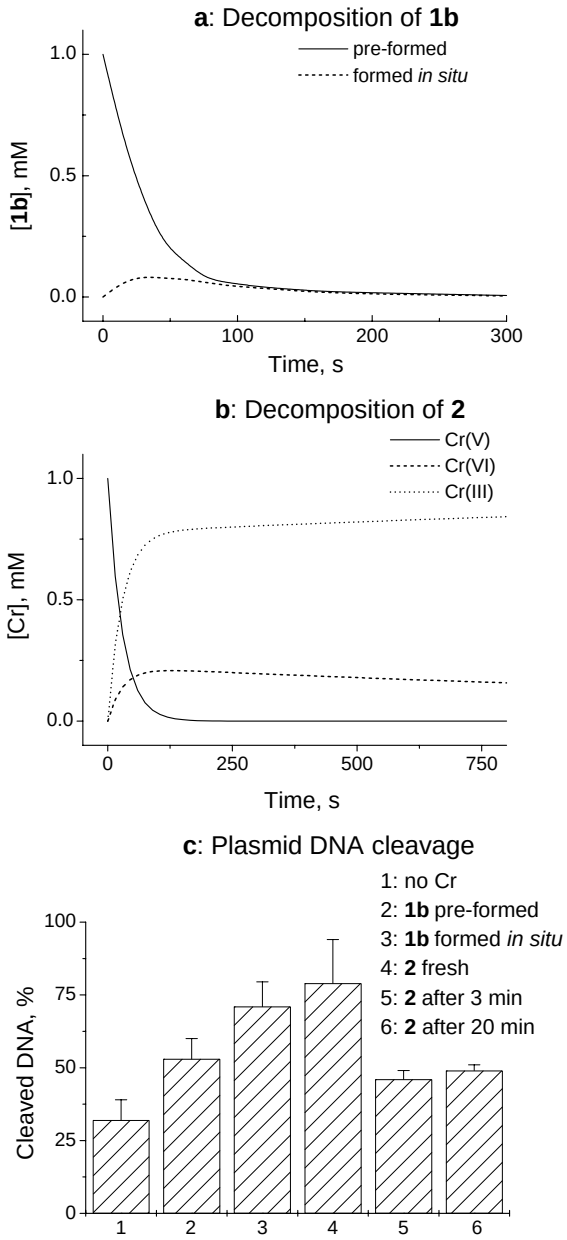
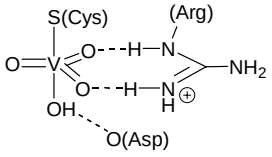


Fig. 6. Decomposition kinetics (a, b) and DNA cleavage studies (c) for complexes **1b** and **2** (based on the data from [133]). Experimental techniques for the isolation (for **2**) or quantitative generation (for **1b**) of the Cr complexes, kinetic analyses (GKA of time-dependent UV-Vis spectra, supported by the results of ESMS and EPR spectroscopy), and plasmid DNA cleavage assays correspond to those described in [27,28,127]. All the reactions were performed in phosphate buffer (0.10 M, pH = 7.4) at $[Cr]_{total}$ = 1.0 mM and 37 °C. Solid line in (a): **1b** was formed in the reaction of $[CrO_4]^{2-}$ (10 mM) with GSH (20 mM) and CF_3COOH (10 mM) in H_2O (pH = 2.9) for 90 s at 4 °C [28], and then diluted 10-fold with the buffer. Dashed line in (a): $[CrO_4]^{2-}$ (1.0 mM), GSH (2.0 mM), and CF_3COOH (1.0 mM) were added to the buffer. Shown in c are the average results and standard deviations of three independent experiments.

DNA damage, despite its short lifetime in aqueous solutions [133,134]. A similar approach, combining kinetic analyses and DNA cleavage assays, has been used to show that oxidative DNA cleavage induced by a model Cr(V) complex,



10

Plate 3. Structure of the V(V) center in vanadate-inhibited protein tyrosine phosphatase [139].

3, in neutral aqueous solutions is due to direct reactions of DNA with **3** [75], rather than with Cr(IV) intermediates formed in its decomposition, as suggested previously [135].

The current understanding of possible mechanistic pathways for Cr-induced protein damage is based mainly on the studies using low-molecular-weight substances as models [1]. Thiol groups of Cys residues in proteins are the most likely targets of Cr-related oxidative damage [49], and detailed mechanistic studies have been performed for the reactions of Cr(VI/V/IV) with either free or Zn-bound biological thiols ([1], and references therein). To date, two classes of proteins have been shown to be susceptible to the loss of function caused by the reactions with Cr(VI/V) complexes. Biological activity of zinc finger proteins (ZnFPs), a family of transcription factors critically important in DNA replication, is dependent on the formation of Zn(II)–S(Cys) bonds ([88], and references therein). Reactions of ZnFPs with the Cr(VI) + GSH system, or with a model Cr(V) complex, **3**, led to the loss of DNA-binding ability of the proteins [88,136,137]. Protein tyrosine phosphatases (PTPs), a large class of enzymes involved in cell signalling, contain a highly conserved $Cys(X)_5Arg$ motif (where X is any amino acid) [138]. The well-known inhibition of PTPs by vanadate is based on the formation of a stable five-coordinate V(V) thiolato complex (**10** in Plate 3, characterized by X-ray crystallography), which acts as a mimic of a transition state of phosphate in these enzymes [139]. The structure of **10** is reminiscent of that of an aquated Cr(VI) thiolato complex, **1b** (Scheme 1); this similarity is undoubtedly related to the isoelectronic nature of V(V) and Cr(VI) ions [28]. Recent experiments demonstrated the ability of Cr(VI) (as well as of a Cr(V) complex, **3**) to cause inhibition of an isolated microbial PTP [12]. Inhibition of PTPs in Cr(VI)-treated mammalian cells is likely to be responsible for the experimentally observed increased levels of tyrosine phosphorylation in such cells [23,140], and for the insulin-mimetic activity of Cr(VI) (similar to that of V(V)) [21–24].

5. Concluding remarks

Improvements in the techniques of kinetic analyses, on the one hand, and in the methods of characterization of unstable Cr complexes (such as **1b** or **2**), on the other, allowed the development of more detailed proposed mechanisms of Cr(VI) reductions. While such mechanisms in the past have concentrated on merely the sequences of changes in Cr oxidation states ([6], and references therein), we are now in a position to assign the step-by-step changes in the structures of reactive intermediates for these reactions [47,75]. Structures of some extremely short-lived Cr(V/IV) complexes (e.g., those in Scheme 6a) could be deduced from the structures of Cr(III) products formed in Cr(VI) reductions [47]. Despite these developments, there are still many "missing links" in the mechanisms of Cr(VI) reductions to Cr(III) under biologically relevant conditions. Further progress in the mechanistic studies of biological Cr(VI) reductions is likely to be determined by structural characterizations of Cr(VI/V/IV) complexes with biomolecules.

The existing mechanistic evidence points to Cr(V) as the main, if not ultimate, reactive species in Cr(VI)-induced genotoxicity [1,6,8,55]. Studies with the use of biologically relevant model Cr(V) complexes, such as **3**, showed that this oxidation state is capable of: (i) direct redox reactions with DNA [75,127]; (ii) decomposition with the formation of cationic Cr(III) products that readily bind to DNA [93,141]; (iii) oxidation of biologically important Zn(II) thiolato complexes (while Cr(VI) reacts only with free thiols) [88]; and (iv) triggering chain reactions with the participation of biological reductants and O_2 [25]. Ubiquitous biological ligands, carbohydrates and glycoproteins, are capable of stabilizing Cr(V), but not Cr(IV) [8,84]. This difference, together with generally lower stability of Cr(IV) complexes (compared with their Cr(V) counterparts) in neutral aqueous solutions [84], is likely to diminish the roles of Cr(IV) complexes as genotoxic species. Nevertheless, there are at least two areas of potential biological importance of Cr(IV) complexes that require further research: (i) possible involvement of relatively stable Cr(IV)–protein intermediates in the formation of protein–Cr(III)–DNA cross-links [85]; and (ii) stabilization of Cr(IV) intermediates by picolinato (pyridine-2-carboxylato) ligands [84], which may contribute to the reported adverse effects of a popular nutritional supplement, Cr(III) picolinate ([1], and references therein). Formation of Cr(VI) complexes (such as **1b**) with GSH and other cellular thiols is likely to contribute to the stabilization of high oxidation states of Cr in cells [49], and to cause alterations in cell signalling (through the inhibition of PTPs, Section 4) [12,28], which may trigger a chain of events leading to carcinogenesis [142].

While evidence is increasing for the feasibility of biological Cr(III) oxidation to Cr(VI) (involving the formation of reactive Cr(V/IV) intermediates), little is known about the possible mechanisms of such oxidations (Section 3). Further studies in this area may provide a unified explanation to the seemingly opposite biological effects of Cr(VI) and Cr(III) complexes [1,12].

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