Oxidative Cleavage of Peptide and Protein Disulphide Bonds by Gold(III): a Mechanism for Gold Toxicity

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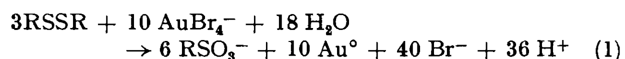
Summary The oxidative cleavage of disulphide bonds to form the sulphonic acid derivatives is shown to be an important reaction of AuX_4^- (X = Cl, Br) with proteins and peptides; the implications of this reaction for the toxicity of gold compounds are discussed.

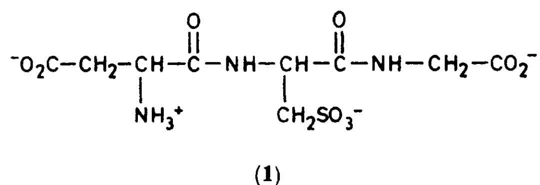
GOLD compounds are an important and effective therapy for rheumatoid arthritis (chrysotherapy), and significant progress has been reported recently in the chemistry of the clinically used drugs and related compounds.[1,2] Thiolate ligands are important as medicinal carrier-ligands for the therapeutically useful oxidation state gold(I), and are probably the principal binding sites for gold(I) in serum, kidney cytosol, and other tissues.[2,4] In contrast, gold(III) compounds are highly toxic and therefore not suitable for drug use.[1-7] The oxidation of thiols to disulphides[6] and the oxidation of methionine to methionine sulphoxide[8] have been suggested to contribute to the toxicity of gold(III). Gold(III) complexes are more potent enzyme inhibitors than gold(I) thiolates.[1] Serum albumin is capable of reducing gold(III) to metallic gold.[2]

It was recently reported that gold(III) oxidatively cleaves disulphide bonds of cystine, homocystine, and penicillamine disulphide, forming the sulphinic and ultimately the sulphonic acid derivatives with concomitant deposition of metallic gold [reaction (1)].[9] Using glutathione, vasso-



$$3RSSR + 10\,AuBr_4^- + 18\,H_2O \rightarrow 6\,RSO_3^- + 10\,Au^\circ + 40\,Br^- + 36\,H^+ \quad (1)$$

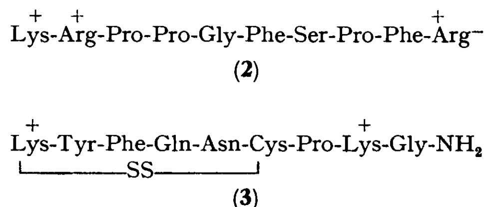
pressin, and insulin as model systems, we now present evidence that cleavage of disulphide bonds is an important reaction of gold(III) complexes with peptides and proteins. Glutathione disulphide, GtSSGt, (0·135 mmol) in 2 ml of H_2O was treated with $KAuBr_4{\cdot}2H_2O$ in molar ratios of 0·67, 1·00, 2·00, and 3·33 with respect to GtSSGt, the latter corresponding to the stoicheiometry of equation (1). The solutions began to decolourize immediately and the deposition of metallic gold began within a few minutes. After 4 h the gold was filtered off and dissolved in *aqua regia*, and its amount determined by atomic absorption spectroscopy. Recoveries were 82, 99, 80, and 79%, respectively. Aliquots (4 μl) of the filtrates were subjected to paper electrophoresis at 50 V/cm for 30 min in H_2O–pyridine–acetic acid (900:100:3·5 v/v) buffer, pH 6·5, using GtSSGt and GtSH as standards. After staining with ninhydrin solution, GtSH and GtSSGt were found to have moved 27 and 33 mm towards the anode, while the oxidation product of the 3·33:1 reaction moved 57 mm, with some tailing. The approximately doubled mobility of the reaction product, compared to GtSH and GtSSGt which have similar q/m ratios, is consistent with oxidative cleavage of the disulphide to form the sulphonic acid derivative (**1**), which is deprotonated at



(**1**)

pH 6·5 giving a net −2 charge per glutathione moiety. ^{13}C N.m.r. studies were also conducted on glutathione disulphide–$KAuBr_4$ reaction mixtures run in phosphate-buffered D_2O at pD 7·4. The β-carbon resonance of the cysteine residue shifted downfield from 28·2 and 42·3 p.p.m. (*vs.* 3-trimethylsilylpropionic acid) in GtSH and GtSSGt,[10] to 54·79 p.p.m. in (**1**), corresponding closely to the shift observed for the β-carbon of cystine in the same reaction.[9]

Tailing of the product of reaction between $KAuBr_4$ and cystine, previously shown to be cysteic acid,[9] was also observed upon electrophoresis. Therefore, a reaction between $KAuBr_4$ and lysine bradykinin (**2**), which has a +3 charge and no cysteine or methionine was examined as a control. No reduction of $KAuBr_4$ occurred but some tailing of intact (**2**) was observed during electrophoresis, establishing that the non-sulphur amino acids and the amide bonds do not reduce gold(III) but interact with it to cause the tailing.



When the products of the reaction between $KAuBr_4$ and vassopressin (**3**) [80 μl of 10·4 mM-(**3**) with molar ratios of 3·33 and 1·11 in 0·1 M-phosphate buffer; pH 7·4; 4·5 h] were subjected to electrophoresis, the product of the stoicheiometric reaction did not migrate, consistent with the introduction of SO_3^- groups into (**3**) by oxidation of the disulphide moeity. The only product of the 1·11:1 molar ratio reaction mixture had an electrophoretic mobility between that of (**3**) and fully oxidized (**3**), suggesting the introduction of one negatively charged group, a mechanism for which is proposed below.

Insulin has an ideal structure for the study of disulphide cleavage, since it consists of two peptide chains, A with 21 residues and a net negative charge and B with 30 residues and a net positive charge, which are joined by two interchain disulphide bonds. A third disulphide bond is internal to the A-chain and there are no methionine or reduced cysteine residues. Complete oxidative cleavage of the disulphides should produce the A- and B-chains in oxidized (sulphonate) form. When bovine insulin (5·3 μmol) was treated with $KAuBr_4$ (10:1 molar ratio) in phosphate buffer, pH 2·5, the solution immediately began to decolourize and turned cloudy. After 6 h metallic gold was present and the solution was transparent indicating that complete reduction of the gold(III) had occurred. The reaction mixture, native insulin, and authentic oxidized A-chain were subjected to electrophoresis for 30 min at 50 V/cm in buffer (aqueous 4 M-urea, 15% formic acid, 10% acetic acid; pH 1·9),[11] then stained with Pauley's reagent,[12] and developed with Na_2CO_3 solution (Figure 1). The A-chain formed during the reaction produced a clear spot corresponding to the authentic sample. A weak spot was sometimes obtained in the appropriate region for the B-chain (we were unable to locate a commercial source of B-chain for comparison). Formation of the oxidized A-chain and the less soluble B chain unequivocally demonstrated that oxidative cleavage of protein disulphides by gold(III) was occurring. Under similar conditions, oxidized A-chain (Sigma Chemical Co.) did not reduce $KAuBr_4$ and was not altered in electrophoretic mobility, substantiating the role of the disulphide bond as the active reducing agent.

Gel-exclusion chromatography was used to study further several $KAuBr_4$–insulin reaction mixtures (10:1 and 30:1 molar ratios; 1·75 μmol insulin in 8 M-urea to solubilize

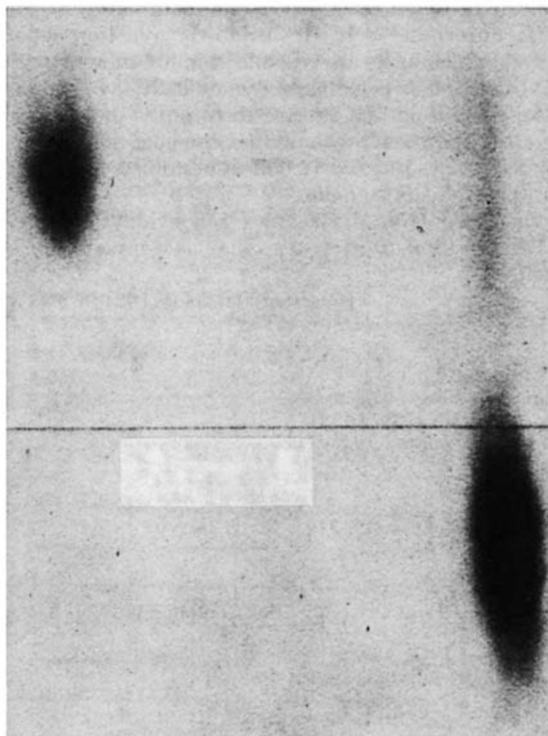


Figure 1. High voltage paper electrophoresis of the $KAuBr_4$–insulin reaction products. From the left, authentic oxidized A-chain and the reaction products (10:1 molar ratio). Aliquots (5 μl) were subjected to 50 V/cm for 30 min in pH 1·9 buffer (4 M-urea, 15% formic acid, 10% acetic acid) for 30 min, and stained with Pauley's reagent developed with Na_2CO_3 solution.

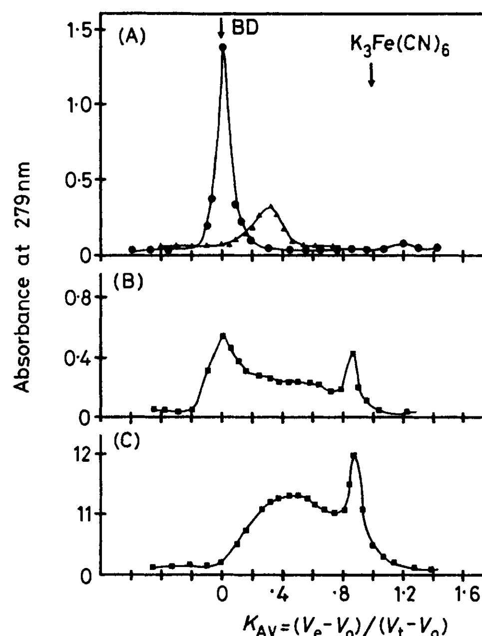
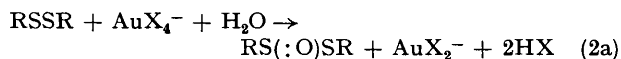


Figure 2. Gel exclusion chromatography of insulin and its gold cleavage products. (A) Bovine insulin (●) and oxidized A-chain (▲) chromatographed separately. (B) The products of the 10:1 molar ratio reaction of $KAuBr_4$ and insulin (■). (C) The products of the 30:1 molar ratio reaction of $KAuBr_4$ and insulin (■). Metallic gold was recovered in 79 and 45% yield for (B) and (C), respectively, and some insoluble protein had precipitated. Samples of the filtrate were fractionated and compared with the calibration standards: Blue-Dextran-2000 (BD), insulin, oxidized A-chain, and $K_3Fe(CN)_6$. A Sephadex G-25-80 (1·5 × 48 cm) column was eluted with 1 M-HOAc–50 mM-phosphate buffer, pH 2·5, at a flow rate of 20 ml/h.

the oxidized A- and B-chains) (Figure 2). Again A-chain was clearly present, along with a second lower-molecular weight fragment which may be B-chain (Figure 2). When a 1:1 molar ratio of $AuCl_4^-$ was employed, reduction was also rapid as shown by the loss of colour, but no oxidized A-chain was present (not shown). At a 4:1 ratio, some A-chain and a small amount of a heavier fragment were present, along with uncleaved insulin. In both cases some gold eluted in the insulin peak; in the 1:1 case it corresponded to 20% of the gold used. In neither reaction did any metallic gold deposit, suggesting that a gold(I) species was the reduction product.

To explain the binding of gold to insulin and the introduction of a single negatively charged group into (**3**) (*vide supra*) when the AuX_4^-: disulphide ratio was below the stoicheiometry of equation (**1**). we propose that a two-electron oxidation of cystine to the disulphide monoxide took place, followed by hydrolytic cleavage to form cysteine sulphinic acid and a cysteine residue [reactions (2a) and (2b)]. These reactions have ample precedent in the aqueous



$$\mathrm{RSSR + AuX_4^- + H_2O \rightarrow RS(:O)SR + AuX_2^- + 2HX} \quad (2a)$$

$$\mathrm{RS(:O)SR + H_2O \rightarrow RSO_2^- + RSH + H^+} \quad (2b)$$

chemistry of cystine,[13] and provide a explanation for the +1 charged product formed in the 1·11:1 $KAuBr_4$ reaction with (**3**), since the sulphinic acid would dissociate while the thiol does not. The free thiol group released in reaction (2b) is able to bind gold(I) and stabilize it, perhaps forming a complex analogous to CySAuCl recently isolated by Brown *et al.* [reaction (3)].[14] Formation of an insulin–gold complex

$$\mathrm{RSH + AuX_2^- \rightarrow RSAuX^- + HX} \quad (3)$$

via reaction (3) explains why gold coeluted with insulin reaction products having the same molecular weight as the native protein after treatment with 1 or 4 mol. equiv. of $AuCl_4^-$.

The rate of the insulin–$AuCl_4^-$ reaction was measured under pseudo-first-order conditions ($[AuCl_4^-]$ = 18—37 μM; [insulin] = 750—1875 μM; KCl = 0 and 0·15 M in 1 M-acetate–50 mM-phosphate buffer at pH 2·5 at 25 °C) by following the disappearance of the $AuCl^-_4$ absorption at 310 nm. Under these conditions the reduction of gold(III) to gold(I) was complete in 2—10 min.

The initial periods of the reaction yielded linear semi-logarithmic plots of $A_t - A_\infty$ *vs. t*, for which the pseudo-first-order rate constant was dependent on the insulin concentration. From these data, a second-order mechanism was deduced: rate = k_2[insulin]$[AuCl_4^-]$ where $k_2 = 7·9 \pm 0·8$ l mol^{-1} s^{-1} for reaction in the absence of added chloride. The reaction with $KAuBr_4$ was too rapid to be followed by conventional u.v.–visible spectroscopic techniques.

As demonstrated by these results, the oxidation of disulphide bonds in peptides and proteins by gold(III) is a rapid

and irreversible reaction, resulting in denaturation of the protein. It may be a principle contribution to the toxicity which precludes the use of gold(III) for chrysotherapy. Indeed, the consequences of disulphide bond cleavage *in vivo* are more deleterious than the reversible oxidation of thiols to disulphides or the conversion of methionine residues into sulphoxides. The oxidation of protein disulphide groups may contribute to the greater inhibition of enzymes by gold(III) than by gold(I). The reported reduction of AuX_4^- to Au° by serum albumin,[2] which contains seventeen disulphide bridges per molecule, may occur *via* the oxidation of disulphides.

The role of redox reactions in the metabolism of gold(I) drugs is uncertain.[1,2,14] However, the reaction reported here would preclude an appreciable accumulation of gold(III) species *in vivo* since it is thermodynamically favourable and kinetically facile in the reverse direction. Indeed, recent *X*-ray absorption studies show that the gold deposits in rat kidney aurosomes isolated 12 h after administering $NaAuCl_4$ contain predominantly gold(I).[15]

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