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Bis(triethylphosphine)gold(I) Chloride: Ionization in Aqueous Solution, Reduction in Vitro of the External and Internal Disulfide Bonds of Bovine Serum Albumin, and Antiarthritic Activity

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Bis(triethylphosphine)gold(I) chloride (**1**) is an orally active antiarthritic agent in the adjuvant-induced arthritic rat model at doses of 10 and 20 (mg of Au/kg)/day. ^{31}P NMR and conductivity measurements show that **1** ionizes in aqueous solution, forming $[(Et_3P)_2Au^+]$ and Cl^-. **1** reacts with bovine serum albumin (BSA) at cysteine-34 of mercaptalbumin (AlbSH), producing $AlbSAuPEt_3$ and free Et_3P, which reduces both the "external" disulfide bonds between Cys-34 and free cysteine or glutathione and some of the "internal" disulfide bonds of the albumin tertiary structure. The cysteines generated from the internal disulfide bonds can be titrated with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and also react with Et_3PAuCl, forming $(Et_3PAu)_x(HS)_{2-x}BSA$. The ^{31}P NMR chemical shifts of Et_3PAu^+ bound at Cys-34 and the newly reduced "internal" cysteines can distinguish the two sites: δ_P = 38.8 ppm for $AlbSAuPEt_3$ and 35–36 ppm for $(Et_3PAuS)_x(HS)_{2-x}BSA$, relative to internal trimethyl phosphate (TMP). Modified albumin samples, in which the cysteine-34 residue is converted into a cysteine disulfide (Cy-BSA) or a thioether (Ac-BSA, prepared by using ICH_2CONH_2), also react with **1**. The mercaptalbumin adduct is extensively regenerated from Cy-BSA by reduction of the "external" disulfides. For Ac-BSA, the regeneration of AlbSH is limited by the extent that the naturally occurring disulfides of cysteine and glutathione were present before the modification. After saturating the free sulfhydryl groups of albumin, Et_3PAuCl also populates weaker binding sites characterized by chemical shifts between 28 and 23 ppm and previously shown to be nitrogen donor groups. Simultaneously, the $AlbSAuPEt_3$ moiety is reversibly transformed into a new species characterized by a resonance at 36 ppm and assigned as $AlbS(AuPEt_3)_2^+$.

Introduction

Gold-based drugs are an important treatment for rheumatoid arthritis, although their mechanism or mechanisms of action are, as yet, undeciphered. Recent progress in the molecular pharmacology of gold drugs[2] has been complemented by insights into their chemical properties and the biochemistry of their reactions with various proteins.[3,4] Serum albumin is a de facto carrier of gold in the blood. Previous studies on the reactions of gold compounds with albumin have identified cysteine-34 of albumin as the principal binding site of gold(I).[5,6] This unusual thiol has an affinity for gold(I) greater than that of other protein and non-protein thiols.[7,8]

Sadler et al. first observed the formation of triethylphosphine oxide from the triethylphosphine ligand of auranofin ((2,3,4,6-tetra-*O*-acetyl-β-1-thio-D-glucopyranosato-*S*)(triethylphosphine)gold(I)) in the presence of albumin.[9,10] This in vitro reaction models (and indeed may be a mechanism for) the in vivo generation of Et_3PO in auranofin-treated patients and laboratory animals.[7] The in vitro generation of triethylphosphine oxide from auranofin analogues, Et_3PAuSR, in the presence of albumin is fastest for the thiolate ligands (RS^-) that have the greatest affinities for gold. A mechanism involving displacement of phosphine from the albumin–gold adduct, $AlbSAuPEt_3$, by thiols has been proposed to explain the data.[7] During an investigation into the ability of cyanide to drive Et_3PO formation, it became clear that redistribution of the ligands in Et_3PAuCN to form $(Et_3P)_2Au^+$ might be an important step in an alternative mechanism for the reaction.[11] Sadler reported that excess $(Et_3P)_2AuCl$ denatured albumin with concomitant formation of Et_3PO,[9] but did not study the reaction with smaller $(Et_3P)_2AuCl$ concentrations. Hill and Girard have shown that $(Et_3P)_2AuCl$ reacts with small organic sulfides, producing Et_3PO and/or Et_3PS, depending on the reaction conditions.[12] Therefore, we have undertaken an investigation into the reaction of $(Et_3P)_2Au^+$ with serum albumin, focusing on the cleavage of disulfide bonds, which Sadler proposed to be the oxidants for the phosphine,[9,10] and the nature of the newly generated gold binding sites.

Our interest in these biochemical studies was further stimulated by our finding that $(Et_3P)_2AuCl$, upon oral administration, has biological activity similar to that of auranofin in the adjuvant-induced arthritic rat model (see Results).

Albumin is actually a microheterogeneous mixture of several molecules,[13] and is designated BSA in this manuscript. The single free cysteine at position 34 forms "external" disulfide bonds to glutathione and non-protein cysteine in 30–35% of the molecules in vivo.[13] Thus, native albumin contains three major components, mercaptalbumin (AlbSH) and the two disulfides (AlbSSCy and AlbSSGt). Each of these has 17 "internal" disulfide bonds. Bovine serum albumin, which is strongly homologous to human albumin,[14] was used for this study because the mercaptalbumin content (SH titer) is more reproducible. Two modifications, Ac-BSA and Cy-BSA, in which the mercaptalbumin component is converted

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to a thioether ($AlbSCH_2CONH_2$) and a disulfide (AlbSSCy), respectively, were used to investigate the role of the cysteine-34 and its disulfide adducts in the reaction.

Experimental Section

Materials. Sephadex G-50 and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Biochemicals; BSA (fatty acid free, Lot No. 1074000823) was obtained from Boehringer Mannhein Biochemicals; iodoacetamide-blocked BSA (Ac-BSA) was prepared as previously described;[5] cysteine-blocked BSA (Cy-BSA, Lot No. 11 M) was obtained from Miles Chemical Co.; D_2O was obtained from Aldrich Chemical Co. Et_3PAuCl was prepared as previously described.[15]

$[(Et_3P)_2Au]Cl$. A solution of Et_3PAuCl (3.5 g, 10 mmol) and Et_3P (1.5 mL, 15.6 mmol) in CH_2Cl_2 (50 mL) and anhydrous diethyl ether (100 mL) was stirred overnight, and the volatiles were removed at reduced pressure. The resulting solid residue was recrystallized (2×) from benzene/petroleum ether to give 3.5 g (74%) of product as a white solid, mp 123–125 °C, with a phosphine-like odor. Anal. Calcd for $C_{12}H_{30}AuClP_2$: C, 30.75; H, 6.45; P, 13.22. Found: C, 30.88; H, 6.52; P, 13.60.

^{31}P NMR Measurements. ^{31}P NMR spectra were obtained on a Brucker WM-250 multinuclear NMR spectrometer operating at 101.3 MHz. The chemical shifts were measured and are reported by using trimethyl phosphate (TMP; δ_P = 2.74 ppm relative to external 85% H_3PO_4) as an internal standard. Typical acquisition parameters were 45° pulse, 0.54-s repetition time, and 16K data points. The spectral windows were −30 to +110 ppm. Approximately 5000–10000 scans were accumulated for each NMR measurement. The various albumin solutions were 2–4.5 mM in 100 mM NH_4HCO_3 buffer. Concentrated solutions of $(Et_3P)_2AuCl$ in methanol (100–200 mM) were added to the albumin solutions just before obtaining the spectra. In some cases, Et_3PAuCl was added to the solution to react with the albumin thiols created in situ.

Analyses. Gold was quantitated by flame atomic absorption spectroscopy (AAS), and albumin was quantitated by its UV absorption at 278 nm (ϵ_{278} = 39600 L/(mol cm)). The albumin SH titer was measured by using DTNB. For reaction products, the SH titer and bound gold (Au_b/BSA) were measured after chromatographic isolation of the product using Sephadex G-50.

Reaction of BSA and $(Et_3P)_2Au^+$. A solution of BSA (1.67 mL, 4.04 mM) with an SH titer of 0.66 was treated with successive aliquots of $(Et_3P)_2AuCl$ (200 mM; 34, 34, and 68 μL), obtaining an NMR spectrum after the first two additions. The final addition caused precipitation of the albumin, and no spectrum was detectable.

Reactions of Ac-BSA and $(Et_3P)_2Au^+$. An Ac-BSA solution (2.2 mL, 3.11 mM; SH titer 0.01) was added with successive aliquots of $(Et_3P)_2AuCl$ (200 mM; 34, 34, and 68 μL), obtaining an NMR spectrum after each addition. No precipitation occurred in this system.

Reaction of Cy-BSA and $(Et_3P)_2Au^+$. Successive aliquots (85, 85, and 170 μL) of $(Et_3P)_2AuCl$ (100 mM) were added to a solution of Cy-BSA (4.3 mM, 2.0 mL; SH titer <0.06), obtaining in each case an NMR spectrum. After the second addition, the solution became viscous and some line broadening was observed.

Characterization of Reduced Cy-BSA and Ac-BSA. Otherwise identical solutions of Ac-BSA and Cy-BSA (4.4 mM, 1.98 mL) were stirred with 200 μL of 200 mM $(Et_3P)_2AuCl$ for 1 h. Each protein was separated from low molecular weight species by using a Sephadex G-50 column and then reconcentrated and analyzed for gold, SH titer, and albumin and by ^{31}P NMR spectroscopy. The reduced and reconcentrated Cy-BSA was further treated with three successive aliquots of Et_3PAuCl (42, 55, and 75 μL), obtaining a ^{31}P NMR spectrum and setting aside a 100-μL aliquot for measurement of bound gold after each addition.

Adjuvant-Induced Arthritic Rat Assay. Lewis rats were treated with four different doses of $[(Et_3P)_2Au]Cl$ according to a published procedure.[16] Adjuvant arthritis was produced by single intradermal injection of 0.75 mg of *Mycobacterium butyricum* suspended in white paraffin oil (light N.F.) into the left hindpaw footpads of male rats. The injected paw became inflamed and reached maximal size within 3–5 days (primary lesion). The adjuvant arthritis (secondary lesion) occurred after a delay of approximately 10 days and was characterized by inflammation of the noninjected right hindpaw and further increases in the volume of the injected hind paw. Test compound suspended in a 0.5% tragacanth vehicle was administered po daily beginning on the day of adjuvant injection, for 17 days, exclusive of days 4, 5, 11, and 12. Drug activity on the primary (left paw, day 3) and secondary (both paws, day 16) lesions was determined by comparing paw volumes of the treated groups with a control arthritic (vehicle) group. Hindpaw volumes were measured by immersing the paw into a mercury reservoir and recording subsequent mercury displacement. The percent change in paw volume of 8 treated animals relative to 16 nontreated controls and its significance (determined by Student's *t* test) was calculated for each compound. The ED_{25} value is defined as the calculated dose that produces a 25% decrease in paw volume relative to nontreated controls.

Table I. Antiarthritic Activity of $(Et_3P)_2AuCl$ in the Adjuvant-Induced Rat Assay

dose, (mg/kg)/day (calcd as Au)	% redn of hindpaw vol from adjuvant control[a,b] injected paw (left) day 3	injected paw (left) day 16	uninjected paw (right) day 16	serum gold level, μg of Au/mL
20	−27[c]	−38[c]	−53[c]	8.2
10	−15[d]	−18[d]	−18[e]	3.6
5	−17	−10	0	2.7
2.5	−6	−4	3	2.1

[a] % reduction from adjuvant control = (hindpaw volume of untreated adjuvant control rat − hindpaw volume of treated rat)/hindpaw volume of untreated adjuvant control rat. [b] Results are based on 7–8 rats per test group and 12 rats in the control groups. [c] $P < 0.001$, significantly different from control with use of Student's *t* test. [d] $P < 0.01$. [e] $P < 0.05$.

Table II. ED_{25}[a] Values for $(Et_3P)_2AuCl$ and Auranofin[b]

compd	left hind paw day 3	left hind paw day 16	right hind paw day 16
$(Et_3P)_2AuCl$	21[c] (8–508[d])	12 (7–26)	10 (7–14)
auranofin[b]	24 (13–101)	25 (16–68)	21 (15.4–31.2)

[a] ED_{25} is a calculated effective dose that produces a 25% decrease in paw volume compared to control values. [b] Reference 16. [c] (mg of Au/kg)/day. [d] 95% confidence limits.

Results

Antiarthritic Activity. $(Et_3P)_2AuCl$ was evaluated for antiarthritic activity in an adjuvant-induced arthritic rat model.[16] An inflammatory arthritis is experimentally induced by injecting an oily suspension of *M. butyricum* into the left hindpaw footpad of the rat. Subsequently, the inflammation and arthritis developed in the uninjected (right) hindpaw and other joints. This model of arthritis responds to certain classes of antiarthritic drugs, including chrysotherapeutic (gold-based) agents. Comparison of the left hindpaw volume (i.e. swelling) at day 3 for treated and adjuvant control animals provides a measure of the antiinflammatory activity of a gold complex. Measurement of the right hindpaw swelling at day 16 monitors immunoregulatory activity, as well as antiinflammatory activity.

Lewis rats were treated with four separate doses of $(Et_3P)_2AuCl$ according to a published procedure.[16] Serum gold levels were measured and an ED_{25} value (estimated dose that produces a 25% decrease in paw volumes compared to control animal paw volumes) was determined. The data are shown in Tables I and II. Oral administration of $(Et_3P)_2AuCl$ at doses of 5, 10, and 20 (mg of gold/kg)/day produced a significant dose-response suppression of the primary lesions (left paw, day 3) of adjuvant arthritic rats compared to controls. On day 16 a significant response on the left paw and the secondary lesion (right paw) was seen only at 10 and 20 (mg of gold/kg)/day. No reponse was seen at 2.5 mg of gold. The suppression in paw volumes at 20 mg of gold appears comparable to those observed for auranofin, although the serum gold level of 6.9 μg/mL previously reported for auranofin[17] is lower than 8.2 μg/mL observed for $(Et_3P)_2AuCl$ at that dose. A comparison of ED_{25} values of $(Et_3P)_2AuCl$ with those of auranofin[17] shows similar numbers for the primary lesion (left paw, day 3), but are approximately half that seen on day 16 in both paws (Table II).

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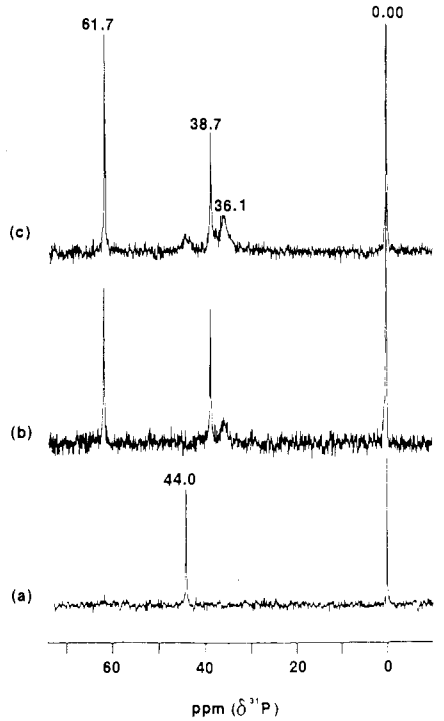

Figure 1. $^{31}P\{^1H\}$ NMR spectra from the reaction of $[(Et_3P)_2Au^+]$ with BSA: (a) 3.4 mM $[(Et_3P)_2Au^+]$; (b) 4.0 mM BSA + 4.0 mM $[(Et_3P)_2Au^+]$; (c) 3.9 mM BSA + 7.8 mM $[(Et_3P)_2Au^+]$. All spectra were obtained in 100 mM NH_4HCO_3 buffer, pH 7.9 with TMP ($(CH_3O)_3P{=}O$) as internal standard. Typically 5000–10 000 scans were accumulated on a Bruker WM-250 spectrometer operating at 101.3 mHz.

Aqueous Solution Structure. The solid-state structure of $(Et_3P)_2AuCl$ may be either ionic with digonally coordinated gold or molecular with trigonally coordinated gold. No crystal structure has been reported to date. However, the gold-197 Mössbauer parameters of solid $(Et_3P)_2AuCl$ obtained at liquid-helium temperature are consistent with an interaction of chloride with gold and an appreciable deviation from linearity, suggesting a trigonally coordinated gold.[18] Conductivity measurements on aqueous solutions of 1 mM $(Et_3P)_2AuCl$ gave a molar conductivity (Λ_M) of 93.0 Ω^{-1} cm^2, similar to that for Et_4NBr obtained under the same conditions.[19] These values are consistent with a 1:1 electrolyte in aqueous solution. The ^{31}P NMR spectrum obtained for an aqueous solution (Figure 1a) has a single resonance at 44 ppm, identical with that of the ionic compound $[(Et_3P)_2Au]NO_3$. Thus, from the chemical shift and conductivity data, it can be concluded that the chloride complex ionizes in aqueous solution, regardless of its solid-state structure. In discussing the reactions with albumin described below, the species actually present in solution, $(Et_3P)_2Au^+$, will be invoked.

Protein Chemistry. When 1 equiv of $(Et_3P)_2Au^+$ is allowed to react with BSA having an SH titer of 0.66, ^{31}P NMR resonances attributable to Et_3PO (61.7 ppm[5,7,9,10]) and AlbSAuPEt$_3$ (38.8 ppm[5,7,9,10,20]) and a new one at 36.1 ppm appear within the time required to obtain the spectrum (Figure 1b). The first two products can be explained by the following reactions. First, the cationic gold complex undergoes a ligand-exchange reaction at cysteine-34 of mercaptalbumin:

$$AlbSH + (Et_3P)_2Au^+ \rightarrow AlbSAuPEt_3 + PEt_3 + H^+ \quad (1)$$

Next, the phosphine liberated by that reaction reduces one of the many disulfide bonds of albumin. This reaction is not limited to mercaptalbumin, but can occur at any of the albumin components, represented as BSA:

$$\overline{S_2}BSA + PEt_3 + H_2O \rightarrow Et_3PO + (HS)_2BSA \quad (2)$$

^{17}O NMR and GC-MS evidence that water ($H_2{}^*O$) is the principal source of the oxygen incorporated into $Et_3P{}^*O$ and that the SH titer of albumin increases has been presented.[21] The reduced albumins, $(HS)_2BSA$, are potentially a family of microheterogeneous products since the same disulfide bond (among the 17 available) may not be reduced in each albumin molecule. The 36.1 ppm resonance is in the region characteristic of thiolate complexes, Et_3PAuSR,[7,20] and can be explained by the binding of Et_3PAu^+ at the newly generated thiol residues of $(HS)_2BSA$:

$$(HS)_2BSA + x(Et_3P)_2Au^+ \rightarrow (Et_3PAuS)_x(HS)_{2-x}BSA + xEt_3P \quad (3)$$

Although the affinity of the cysteine-34 residue for gold is unusually great,[7,8] its limited availability (0.66 AlbSH/BSA) causes it to saturate, so that any additional gold must bind at other, lower affinity sites. This prediction was confirmed when an additional equivalent of $(Et_3P)_2Au^+$ was added (Figure 1c). The intensities of the Et_3PO and $(Et_3PAuS)_xBSA$ resonances, but not the AlbSAuPEt$_3$ resonance, increased. In addition, a weak resonance at 44.0 ppm, due to $(Et_3P)_2Au^+$, was present.

To gain further insight into the protein chemistry, we investigated the reaction of iodoacetamide-blocked albumin, Ac-BSA. The iodoacetamide reacts with mercaptalbumin, converting cysteine-34 into a thioether derivative (AlbSCH$_2$CONH$_2$). The disulfide components of the albumin, however, are unaffected. This modification has proven useful in studying the reactivity of auranofin, aurothiomalate, and related gold complexes with albumin.[5,8] When 1 equiv of $(Et_3P)_2Au^+$ was added to a sample of Ac-BSA, a strong resonance at 43.8 ppm, $[(Et_3P)_2Au^+]$, and a weak one at 62.0 ppm, Et_3PO, suggested that very little reaction had taken place (Figure 2a). During the time required to add a second equivalent and remeasure the spectrum, extensive reaction took place (Figure 2b). The $(Et_3P)_2Au^+$ (43.8 ppm) had been largely converted into $(Et_3PAu)_xBSA$ (35.8 ppm), Et_3PO (62.0 ppm), and a small amount of AlbSAuPEt$_3$ (38.8 ppm). The generation of AlbSAuPEt$_3$ from the sulfhydryl-modified albumin,

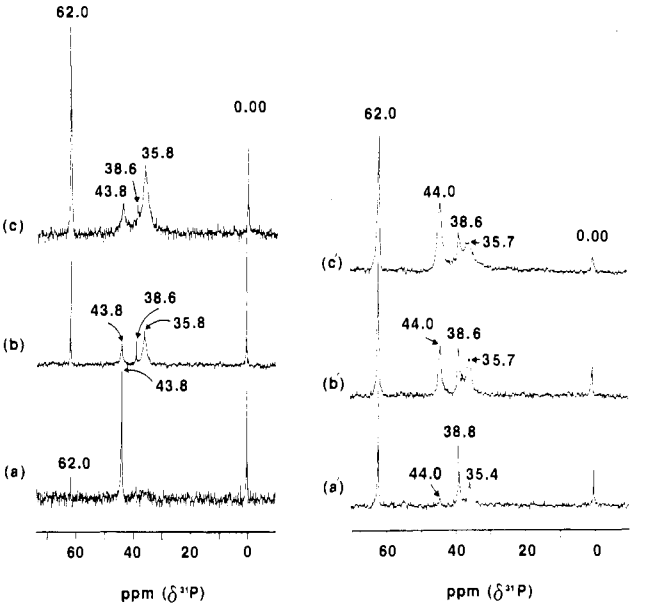

Figure 2. $^{31}P\{^1H\}$ NMR spectra comparing the reactions of Ac-BSA and Cy-BSA with $[(Et_3P)_2Au^+]$: (a) Au:Ac-BSA = 1:1; (b) Au:Ac-BSA = 2:1; (c) Au:BSA = 4:1; (a') Au:Cy-BSA = 1:1; (b') Au:Cy-BSA = 2:1; (c') Au:Cy-BSA = 4:1. The gold complex was added sequentially to 3.1 mM Ac-BSA or 4.1 mM Cy-BSA. Other conditions as in Figure 1.

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Table III. Sulfhydryl and Gold Content of Albumin Reaction Products

reactants albumin; concn, mM	albumin product		
	Au_i/BSA	SH/BSA	Au_b/BSA
Ac-BSA; 1.3	4.5	0.73	2.09
Cy-BSA; 1.9	4.5	1.23	1.89

Ac-BSA, can be explained by reduction of the naturally occurring cysteinyl-34 disulfides of glutathione and cysteine (AlbSSGt and AlbSSCy, respectively), which comprise about 30–35% of the albumin molecules:[13]

$$\text{AlbSSR} + (Et_3P)_2Au^+ \xrightarrow{H_2O} \text{AlbSAuPEt}_3 + Et_3PO + RSH \quad (4)$$

The increase in product formation between parts a and b of Figure 2 is much greater than would be expected for the elapsed time interval and the additional $(Et_3P)_2Au^+$. An explanation can be found in eq 2 and 3, which demonstrate the classical conditions for an autocatalytic chain reaction. The reduction of a disulfide bond generates two thiols. These, in turn, can react with $(Et_3P)_2Au^+$ to release additional Et_3P to continue the branching chain reaction. After Figure 2b was obtained, adding 2 equiv more (4 equiv total) of $(Et_3P)_2Au^+$ increased the amount of Et_3PO and $(Et_3PAuS)_x$BSA, but did not increase the 38.6 ppm resonance (Figure 2c).

To test the assignment of the 38.6 ppm resonance in Figure 2b,c to $AlbSAuPEt_3$, we also studied the reactions of an albumin sample modified by conversion of the mercaptalbumin into a cysteine disulfide of Cys-34, AlbSSCy. In this preparation, in Cy-BSA, which also includes the endogenous disulfide of glutathione (AlbSSGt), all of the cysteine-34 residues are present as disulfides. As shown in Figure 2a′, the Cy-BSA reacts with an equivalent of $(Et_3P)_2Au^+$, yielding more $AlbSAuPEt_3$ than $(Et_3PAuS)_x$BSA, consistent with the increased AlbSSR content. Adding an additional 1 and then 2 equiv of $(Et_3P)_2Au^+$ generates more Et_3PO and $(Et_3PAuS)_x$BSA but no more $AlbSAuPEt_3$ (Figure 2b′,c′).

Gel-exclusion chromatography was used to verify that the broad resonance at 35–36 ppm represents gold bound to the newly reduced cysteines of the protein (generated according to eq 2), rather than $CySAuPEt_3$ or $GtSAuPEt_3$ formed from free cysteine or glutathione released (according to eq 4) by cleaving the "external" disulfides, AlbSSCy and AlbSSGt. The protein products were isolated from the reactions of Cy-BSA or Ac-BSA with 4.5 equiv of $(Et_3P)_2Au^+$. If the 35–36 ppm resonance were due to the low molecular weight adducts, $Et_3PAuSCy$ or $Et_3PAuSGt$, this resonance would be absent from the ^{31}P NMR spectrum of the isolated proteins. As shown in Figure 3a,b, this resonance is present in each case, verifying that it is due to protein-bound Et_3PAu^+ and consistent with the assignment as $(Et_3PAuS)_x$BSA.

For every $(Et_3P)_2Au^+$ that reacts according to eq 1, one Et_3PAu^+ should bind to an albumin sulfhydryl group and one Et_3P should be oxidized, creating two new sulfhydryl groups. Thus, the reduced albumins should have an increased SH content. The isolation of the reduced proteins provided an opportunity to measure their SH titer and the amount of gold bound to them (Table III). From atomic absorption spectroscopy, about 2 equiv of gold were determined to be bound in each case. DTNB was used to measure the free thiol content of the products. Any gold-bound thiols are effectively masked and react very slowly with DTNB.[22] Thus, the values of 0.7 SH/Ac-BSA and 1.3 SH/Cy-BSA represent free thiols in excess of those bound to gold. The sum of the SH titer and the bound gold provides a lower limit for the total number of thiols created: 2.8/Ac-BSA and 3.1/Cy-BSA. (The extent of reduction may be larger than these estimates, because some of the bound gold may be coordinated to two thiols (S–Au–S) and because the glutathione and free cysteine originally present as disulfides of Cys-34 are removed during the chromatographic isolation of the protein.)

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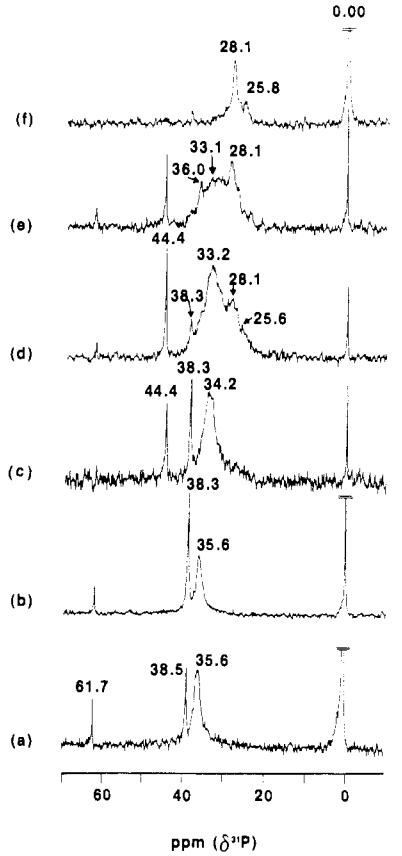

Figure 3. (a, b) $^{31}P\{^1H\}$ NMR spectra of chromatographically isolated albumin after (a) 1.3 mM Ac-BSA or (b) 1.9 mM Cy-BSA was reduced with $[(Et_3P)_2Au^+]$ in a 2:1 ratio. (c–e) Spectrum of the sample from spectrum b titrated with Et_3PAuCl at Et_3PAuCl:Cy-BSA = (c) 1.1, (d) 2.6, and (e) 3.3. (f) Comparison spectrum of Cy-BSA reacted directly in a 1:1 ratio with Et_3PAuCl. Other conditions are as in Figure 1.

The free thiols of the reduced albumins of Figure 3a,b should be able to bind more gold(I), if it is provided in a sufficiently reactive form. Adding successive aliquots of Et_3PAuCl to the reduced Cy-BSA used to obtain Figure 3b generated the spectra shown as Figure 3c–e. The first aliquot (Figure 3c) intensified and broadened the 35–36 ppm resonance, shifting its center to higher field, 34 ppm. At the same time a peak at 44.4 ppm appeared (although Et_3PAuCl, not $(Et_3P)_2Au^+$, was being added). When the next aliquot was added, the 34 ppm resonance broadened and shifted further to 33 ppm, the 38.6 ppm resonance of $AlbSAuPEt_3$ decreased, and new resonances appeared at 28 and 25 ppm. The final aliquot further increased the 28 and 25 ppm resonances. A new resonance, sharper than the broad envelope at 33–38 ppm, emerged at 36.0 ppm, while the 38.6 ppm resonance of $AlbSAuPEt_3$ disappeared.

The 28 and 25 ppm signals were previously assigned to Et_3PAu^+ bound at histidine, and possibly methionine, residues.[5] Consistent with that interpretation, they can be generated directly by reaction of Cy-BSA and Et_3PAuCl, i.e., without any preceding reduction of disulfide bonds (Figure 3f). The $(Et_3P)_2Au^+$ (44.4 ppm) in Figure 3c–e must be formed via ligand exchange reactions. Two possible mechanisms are (1) chelation of protein-bound gold by a second amino acid residue, releasing free Et_3P that extracts an Et_3PAu^+ moiety from the protein, and (2) ligand redistribution (scrambling) reactions. The two descriptions are neither mutually exclusive nor contradictory. The loss of the $AlbSAuPEt_3$ resonance at 38.3 ppm and the simultaneous appearance of the new 36.0 ppm signal depicted in Figure 3c–e are attributed to reaction 5.

$$\text{AlbSAuPEt}_3 + Et_3PAuCl \rightarrow \text{AlbS}(AuPEt_3)_2^+ + Cl^- \quad (5)$$

A similar reaction was postulated previously to explain a 36.0 ppm resonance that appeared with loss of the 38.6, 28, and 25 ppm

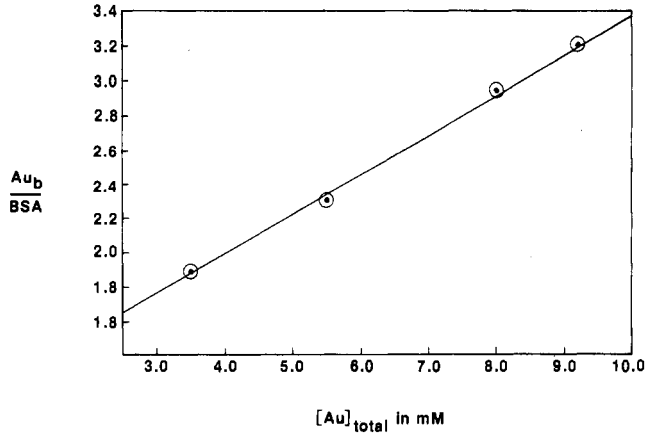

Figure 4. Plot of gold-to-protein ratios after the samples used for Figure 3b–e were chromatographed. The Au_b:Cy-BSA ratio is plotted against the total gold ($[(Et_3P)_2Au^+]$ + Et_3PAuCl) concentration in the solution before chromatography.

resonances after an albumin sample having Et_3PAu^+ bound to Cys-34 and to the nitrogen bases was chromatographed.[5] In that case, the weak (nitrogen) binding sites, rather than the added chloride, were the source of the second Et_3PAu^+ moiety bound at Cys-34.

Measurements of the protein-bound gold (determined by atomic absorption spectroscopy after aliquots of the solutions used to obtain the spectra in Figure 2c–e were chromatographed) revealed an approximately linear dependence of Au_b/BSA on the total gold concentration in the solutions (Figure 4). This result is consistent with extensive binding of the added Et_3PAuCl as Et_3PAu^+ (and possibly some Au^+ as chelated) at various binding sites including the thiols generated by the initial treatment with $(Et_3P)_2Au^+$ and histidine or thioether.

A second set of experiments, combining gel-exclusion chromatography and ^{31}P NMR spectroscopy, suggests that Et_3PAu^+ can dissociate from the weaker binding sites under some conditions. An albumin gold adduct was isolated after Cy-BSA was reduced with $(Et_3P)_2Au^+$ and determined to have gold bound only as $AlbSAuPEt_3$ (38.4 ppm) and $(Et_3PAuS)_xBSA$ (36.0 ppm). Some Et_3PO was present, suggesting that phosphine continues to be displaced from the protein-bound gold during and after the chromatography. After Et_3PAuCl is added to the solution, the weak binding sites characterized by resonances at 36, 31, 28, and 25 ppm are populated, (Figure 5b). Two additional resonances at 23 and 27 ppm are also resolved and assumed to be weak binding sites obscured in the broader envelopes of Figure 3. Et_3PO and $(Et_3P)_2Au^+$, identified by the signals at 62 and 44 ppm, are formed via ligand-exchange reactions. The resulting albumin–gold adduct, which is similar to that in Figure 3e, was isolated via gel-exclusion chromatography to remove the low-molecular-weight reaction products. After concentration of the albumin fractions, the ^{31}P NMR spectrum of in Figure 5c was obtained. Again, the broad, overlapping envelope of peaks between 35 and 23 ppm is present. The partial loss of $AlbS(AuPEt_3)_2^+$ (35.9 ppm) and the regeneration of the $AlbSAuPEt_3$ (38.4 ppm) are noteworthy. This observation suggests that eq 5 is reversed by loss of weakly bound Et_3PAu^+ during the chromatography.

Discussion

The formation of $AlbSAuPEt_3$ by cleavage of the "external" disulfides of albumin and the formation of $(Et_3PAuS)_xBSA$ after reduction of the internal disulfides can be distinguished by the ^{31}P NMR signatures of the two gold species, 38.6 for the former and ca. 35–36 ppm for the latter. The $(Et_3PAuS)_xBSA$ binding sites created from the internal disulfides have chemical shifts typical of Et_3PAuSR complexes, such as those of glutathione, cysteine, and thioglucose. The larger chemical shift value for $AlbSAuPEt_3$ is consistent with the unusually low pK_{SH} value of cysteine-34,[7,8,23] estimated to be as low as ca. 5.[24]

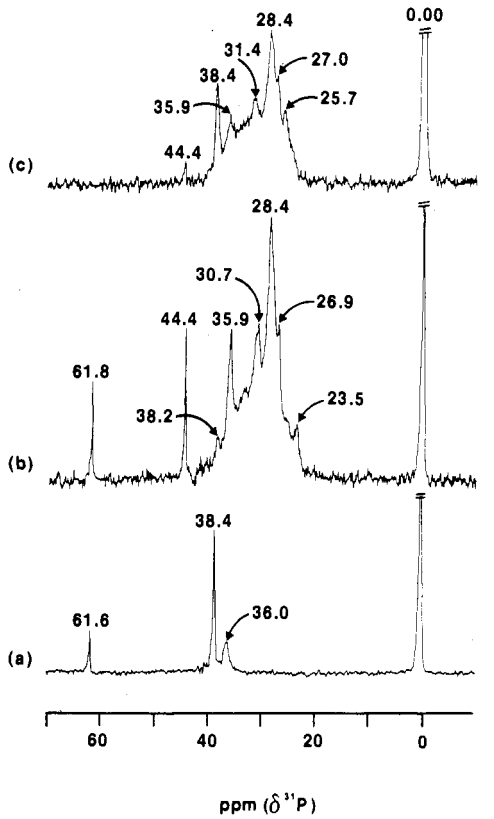

Figure 5. $^{31}P\{^1H\}$ NMR spectra: (a) chromatographically isolated albumin–gold adduct prepared by reducing Cy-BSA with $[(Et_3P)_2Au^+]$ (1.0 mM Cy-BSA, Au_b:BSA = 1.5; (b) sample from part a treated with 7 equiv of Et_3PAuCl; (c) albumin–gold adduct isolated after chromatography of sample from part b (0.95 mM BSA; Au_b:BSA = 8.4). Note the relative changes in the $AlbSAuPEt_3$ and $AlbS(AuPEt_3)_2^+$ peaks between parts b and c. Other conditions are as in Figure 1.

The cys-34 disulfides of AlbSSCy and AlbSSGt are reduced more readily than the internal disulfides of albumin. This is demonstrated by the greater reactivity of Cy-BSA compared to Ac-BSA (contrast parts a and a′ of Figure 2) and by the formation of more $AlbSAuPEt_3$ (38.8 ppm) from the single cysteine-34 disulfide than of $(Et_3PAuS)_xBSA$ (35–36 ppm) from the remaining 17 disulfide bonds in the reaction of Cy-BSA (Figure 2a′). Our experiments can not, however, distinguish whether the difference in reactivity has a thermodynamic or kinetic origin. Since Cys-34 is located in a crevice approximately 1000 pm (10 Å) deep[25] and has an unusually low pK_{SH},[24] both factors may contribute. Albumin is postulated to transport glutathione and cysteine as disulfides of Cys-34. The facile and preferential cleavage of the "external" disulfide bonds observed here is consistent with their postulated roles as glutathione and cysteine transport mechanisms.

Phosphorus donor ligands generally have greater affinities for gold(I) than do thiols. The displacement of Et_3P by cysteine-34 and the cysteines generated by reduction of the disulfide bonds can be explained by a two-step process. First, there is a small but finite concentration of Et_3P displaced from $(Et_3P)_2Au^+$ via a three-coordinate transition state for ligand exchange at gold(I), eq 6. While much of the free phosphine probably reacts im-

$$(Et_3P)_2Au^+ + AlbSH \rightleftharpoons \left[AlbSAu(PEt_3)(PEt_3)\right]^{\ddagger} + H^+ \rightleftharpoons AlbSAuPEt_3 + PEt_3 + H^+ \quad (6)$$

(23) Isab, A. A.; Sadler, P. J. *J. Chem. Soc., Dalton Trans.* **1982**, 135–141.

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(25) (a) Cornell, C. N.; Kaplan, L. J. *Biochemistry* **1978**, *17*, 1750–54. (b) Hull, H. H.; Chang, R.; Kaplan, L. J. *Biochem. Biophys. Acta* **1975**, *400*, 132–136.

mediately in the reverse reaction, oxidation of a small fraction by the disulfide bonds, eq 7, and other oxidants such as O_2 will

$$PEt_3 + RSSR + H_2O \rightarrow 2RSH + Et_3PO \quad (7)$$

gradually shift the equilibrium to the right. Although the net equilibrium of the first step should lie far to the left, the second reaction is irreversible and drives the overall process to completion.

In previous studies of reactions between $[(Et_3P)_2Au]Cl$ and small organic disulfides, the apparent rate-limiting step was dissociation of a phosphine ligand from gold.[12] A similar mechanism apparently operates in the case of the sulfhydryl modified albumins. They initially lack any free sulfhydryls and can not react according to eq 6 and 7. The observation that they do react with $[(Et_3P)_2Au^+]$, although more slowly than does AlbSH, can be explained by eq 8-10. If this sequence were the

$$[(Et_3P)_2Au]Cl \rightleftarrows Et_3PAuCl + Et_3P \quad (8)$$

$$Et_3P + \overset{\frown}{S_2}BSA \xrightarrow{H_2O} (HS)_2BSA + Et_3PO \quad (9)$$

$$Et_3PAuCl + (HS)_2BSA \rightarrow (Et_3PAuS)_x(SH)_{2-x}BSA + H^+ + Cl^- \quad (10)$$

dominant mechanism, the reactions of the modified and unmodified albumins (Cy-BSA and Ac-BSA vs AlbSH) should occur at similar rates. The delay observed with Ac-BSA, in particular (compare Figure 1b and Figure 2a), is consistent with eq 8-10. It further suggests an important role for Cys-34 in the rate-determining step of the AlbSH reaction, as indicated in eq 6.

$[(Et_3P)_2Au]Cl$ differs from auranofin ($Et_3PAuSAtg$) in the physical and chemical properties that might affect its molecular pharmacology. For example, the bis(phosphine) complex is moderately soluble in aqueous solution, while auranofin is only sparingly soluble. Excess $[(Et_3P)_2Au]Cl$ denatures serum albumin,[9] but auranofin does not.[5,9,10] Nonetheless, $[(Et_3P)_2Au]Cl$ generates $AlbSAuPEt_3$ and Et_3PO as the dominant products when it reacts with equimolar or excess albumin (Figure 1b). These are the same products formed in albumin-auranofin reactions.[5] Although the bis(phosphine) complex generates Et_3PO more rapidly and more extensively than auranofin and also forms $(Et_3PAuS)_x(SH)_{2-x}BSA$ as a likely metabolite, the oxide is not medicinally significant and the gold bound to the newly generated, low-affinity cysteines should be at least as active as Cys-34-bound gold. Thus, it is not surprising that $[(Et_3P)_2Au]Cl$ and auranofin have similar activities in the adjuvant-induced arthritic rat assay (Table II).

Elucidation of the sulfur chemistry of serum albumin has been hampered by a lack of suitably sensitive and specific spectroscopic probes. For example, the NMR signals of sulfur-33, the only NMR-active isotope, are too broad to be used for most purposes. We have demonstrated here that Cys-34 and any nonnative cysteines generated by reduction of the internal disulfide bonds can be distinguished by ^{31}P NMR after titration with Et_3PAu^+. This finding provides a new tool for exploring the chemistry of Cys-34 and the internal and external disulfides bonds.

Abbreviations: AAS, atomic absorption spectroscopy; Ac-BSA, BSA modified to convert AlbSH to $AlbSCH_2CONH_2$; AlbSH, mercaptalbumin (a BSA component); AlbSSCy, the cysteine disulfide of albumin Cys-34; AlbSSGt, the glutathione disulfide of albumin Cys-34; BSA, microheterogeneous bovine serum albumin; Cy-BSA; BSA modified to convert AlbSH to AlbSSCy; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); GC-MS, gas chromatography-mass spectroscopy; $(HS)_2BSA$, albumin-containing reduced disulfide bonds; NMR, nuclear magnetic resonance; ppm, parts per million; TMP, trimethyl phosphate.

Registry No. I, 65583-79-1; Et_3P, 554-70-1.

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Magnetic Properties of the Nickel Enzymes Urease, Nickel-Substituted Carboxypeptidase A, and Nickel-Substituted Carbonic Anhydrase

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Magnetic susceptibility measurements have been made on urease and the Ni-substituted forms of carboxypeptidase A (Ni-CPA) and carbonic anhydrase (Ni-CA). The effective magnetic moment per Ni ion in each protein is found to be in the range consistent with six-coordinate Ni(II). Each Ni-protein exhibits low-temperature deviations from the Curie law behavior, and for Ni-CPA and Ni-CA this correlates with axial zero-field splittings the $^3A_2$ ground state of 3.5 and -8.2 cm^{-1}, respectively. The significantly larger magnitude of this deviation observed for urease indicates a magnetic interaction between the two Ni(II) ions in each subunit. Fitting the urease magnetic data indicates a weak antiferromagnetic exchange coupling of the Ni(II) ions ($J = -6.3$ cm^{-1}, using the isotropic exchange Hamiltonian $H = -2JS_1 \cdot S_2$) and an axial zero-field splitting ($D = -6.9$ cm^{-1}) consistent with distorted six-coordinate ligation of the two Ni(II) ions; this experimentally demonstrates for the first time that *urease has a binuclear Ni active site*. A magnetic contribution of single Ni(II) ions, which corresponds to ~20% of the urease Ni, is required for this fit and may be associated with a small heterogeneous subset of sites. The potent competitive inhibitor acetohydroxamic acid increases the urease magnetic moment, indicating a change in the Ni(II) coordination number and/or geometry, and diminishes the low-temperature Curie law deviation, indicating a decrease in the Ni-Ni interaction. Binding of the competitive inhibitor 2-mercaptoethanol (2-ME) results in a diamagnetic Ni(II) ground state for ~80% of the urease Ni ions. The residual moment of this derivative appears to correlate with a percentage of sites where 2-ME apparently does not bind or binds differently than in the dominant ~80% of active sites; this subset of paramagnetic sites may correlate with the native urease sites lacking binuclear interaction. In contrast to urease, 2-ME binding to Ni-CA does not reduce the paramagnetism of the active site Ni(II) ion, suggesting a unique Ni-thiolate interaction for urease. The ligand field absorption spectrum of 2-ME-bound urease does not show intense orbitally allowed transitions associated with a low-spin $^1A_1$ Ni(II) ground state, indicating that the diamagnetism of this inhibitor-bound form appears to be due to a stronger antiferromagnetic exchange interaction between the Ni(II) ions; this further supports a binuclear active site in urease and suggests the possibility of bridging coordination for substrates.

Introduction

Urease (EC 3.5.1.5) catalyses the hydrolysis of urea and was the first enzyme recognized[1] to contain and require Ni for catalytic activity. The enzyme isolated from jack bean has two Ni ions per 96 600-Da subunit and is aggregated into a hexamer of subunits. Urease will hydrolyze a limited number of substrates[2] other

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(1) Dixon, N. E.; Gazzola, C.; Blakeley, R. L.; Zerner, B. *J. Am. Chem. Soc.* **1975**, *97*, 4130-4133.

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