Oxidation of the Phosphine from the Auranofin Analogue, Triisopropylphosphine(2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato-*S*)gold(I), via a Protein-Bound Phosphonium Intermediate

C. Frank Shaw, III,*[1a] Anvarhusein A. Isab,[1a,b] James D. Hoeschele,[1c] Mary Starich,[1a] James Locke,[1a] Pamela Schulteis,[1a] and Jun Xiao[1a]

Contribution from the Departments of Chemistry, The University of Wisconsin—Milwaukee, P.O. Box 413, Milwaukee, Wisconsin 53201-0413, and University of Michigan, Ann Arbor, Michigan 48109

Received August 9, 1993®

Abstract: The reactions of serum albumin, a blood carrier of gold(I), with the auranofin analogue triisopropylphosphine-(2,3,4,6-tetra-*Q*-acetyl-1-thio-β-D-glucopyranosato-*S*)gold(I) (*i*-$Pr_3PAuSATg$) and free triisopropylphosphine have been studied in buffered aqueous solution using $\{^1H\}^{31}P$ NMR and chromatographic methods. Triisopropylphosphine (*i*-Pr_3P) is oxidized to *i*-Pr_3PS via an albuminthiolatotriisopropylphosphonium ion, *i*-$Pr_3P^+SCH_2(HSCH_2)Alb$, which is formed by attack on a protein disulfide bond. This species is the key intermediate in the albumin-driven conversion of a phosphine ligand (e.g., from auranofin or an analogue) into phosphine oxide or phosphine sulfide. *i*-$Pr_3P^+SCH_2(HSCH_2)Alb$, which is characterized by a ^{31}P NMR chemical shift of 75.4 ppm, forms quickly and then reacts slowly ($k_{obs} = (6.9 \pm 0.6) \times 10^{-5}\ s^{-1}$) to form *i*-$Pr_3PS$ and a small quantity of *i*-Pr_3PO. The auranofin analogues *i*-$Pr_3PAuSATg$ and *i*-Pr_3PAuCl, react with serum albumin at cysteine-34 to form $AlbSAuPi\text{-}Pr_3$ via displacement of the anions. *i*-Pr_3PAuCl reacts further at weak binding sites analogous to the histidine binding sites of auranofin. In contrast to the displacement of Et_3P from $AlbSAuPEt_3$ by thiols, cyanide is required to displace *i*-Pr_3P from $AlbSAuPi\text{-}Pr_3$. The liberated *i*-Pr_3P also reacts via the alubminphosphonium intermediate described above to form *i*-P_3PS and traces of *i*-P_3PO. In order to interpret the protein studies, a variety of potential reaction products (*i*-Pr_3PAuX, X = CN, ATgS, Cl; *i*-Pr_3PY, Y = O, S) were prepared and characterized by ^{31}P NMR spectroscopy. Model reactions of *i*-Pr_3PAuX (X = Cl, ATgS) with cyanide are also reported.

Serum albumin acts as a carrier of gold(I) after the administration of gold-based drugs to patients receiving chrysotherapy for rheumatoid arthritis or to laboratory animals.[2,3] Numerous model studies using purified albumin have identified Cys-34 as the critical, high-affinity gold-binding residue,[4-10] and the subject has been reviewed recently.[11] Auranofin [(2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato-*S*)(triethylphosphine)gold(I)], the second-generation gold drug, has unique physiochemical and pharmacological properties, most notably its lipophilicity, due to the presence of a phosphine ligand. The Et_3P is oxidized and excreted as Et_3PO, a nontoxic, water-soluble metabolite of the drug.[12,13] Recent studies from our laboratory have established that albumin–auranofin reactions in vitro model the slow formation of Et_3PO and have properties suggesting that it might be a contributing, and perhaps major, mechanism for the in vivo reaction.[4,5,11,14,15]

The revised crystallographic structure of albumin confirms that the protein is a globular structure organized into three domains, each consisting of two subdomains, and that *Cys*-34 is in domain IA.[16] The protein is microheterogeneous because about 30–40% of the molecules in circulation contain disulfide bonds between Cys-34 and cysteine (AlbSSCy) or glutathione (AlbSSG); the remainder is the so-called mercaptalbumin species (AlbSH) with the fully reduced Cys-34.[17]

Auranofin ($Et_3PAuSAtg$) reacts at Cys-34 via a ligand-exchange reaction in which the acetylthioglucose ligand (ATgSH) is displaced from gold by Cys-34:

$$Et_3PAuSAtg + AlbSH \rightarrow AlbSAuPEt_3 + AtgSH \quad (1)$$

The albumin–gold–phosphine complex is stable if isolated from the displaced thiol.[4,10,18] If the AtgSH remains in solution or is replaced with other thiols, the Et_3P is displaced and oxidized at

* To whom correspondence should be addressed through June, 1994, at Division of Immunology, Medizinisches Institut für Umwelthygeine, Postfach 103751, 40225 Düsseldorf, Germany, and thereafter at Department of Chemistry, The University of Wisconsin–Milwaukee.

® Abstract published in *Advance ACS Abstracts*, December 15, 1993.

(1) (a) University of Wisconsin–Milwaukee. (b) On sabbatical leave from The King Fahd University of Petroleum and Minerals, Dhahran, Saudi Arabia, 1986–87. (c) University of Michigan. Portions of this research were presented at the Pacifichem-89 Meeting, Honolulu, HI, December, 1989. Abstracted in part from the Ph.D. Thesis of J. Xiao, University of Wisconsin–Milwaukee, 1993.

(2) Finkelstein, A. E.; Walz, D. T.; Batista, V.; Mizraji, M.; Roisman, F.; Misher, A. *Ann. Rheum Dis.* **1976**, *35*, 251–257.

(3) Kamel, H.; Brown, D. H.; Ottaway, J. M.; Smith, W. E.; Cottney, J.; Lewis, A. J. *Arthritis Rheum.* **1978**, *21*, 441–446.

(4) Coffer, M. T.; Shaw, C. F., III; Eidsness, M. K.; Watkins, J. W.; Elder, R. C. *Inorg. Chem.* **1986**, *25*, 333–339.

(5) Isab, A. A.; Shaw, C. F., III; Hoeschele, J. D.; Locke, J. *Inorg. Chem.* **1988**, *27*, 3588–3592.

(6) Shaw, C. F., III; Schaeffer, N. A.; Elder, R. C.; Eidsness, M. K.; Trooster, J. M.; Calis, G. H. *J. Am. Chem. Soc.* **1984**, *106*, 3511–3521.

(7) Malik, N. A.; Otiko, G.; Sadler, P. J. *J. Inorg. Biochem.* **1980**, *12*, 317–322.

(8) Malik, N. A.; Sadler, P. J. *Biochem. Soc. Trans.* **1979**, *7*, 731–732.

(9) Gerber, D. A. *J. Pharmacol. Exp. Ther.* **1964**, *143*, 137–140.

(10) Coffer, M. T.; Shaw, C. F., III; Hormann, A. L.; Mirabelli, C. K.; Crooke, S. T. *J. Inorg. Biochem.* **1987**, *30*, 177–187.

(11) Shaw, C. F., III *Comments Inorg. Chem.* **1989**, *8*, 233–267. Berners-Price, S. J.; Sadler, P. J. *Struct. Bonding* **1988**, *70*, 27–102.

(12) Intoccia, A. P.; Flanagan, T. L.; Walz, D. T.; Gutzait, L.; Swagzdis, J. E.; Flagiello, H., Jr.; Hwang, B. Y.-H.; Dewey, R. H. In *Bioinorganic Chemistry of Gold Coordination Compounds*; Sutton, B. M., Ed.; Smith Kline & French Laboratories: Philadelphia, PA, 1983; pp 21–36.

(13) Walz, D. T.; Griswold, D. E.; DiMartino, M. J.; Bumbier, E. E. *J. Rhematol.* **1980**, *7*, 820–824.

(14) Hormann, A. L.; Shaw, C. F., III; Bennett, D. W.; Reiff, W. *Inorg. Chem.* **1986**, *25*, 3953–3957.

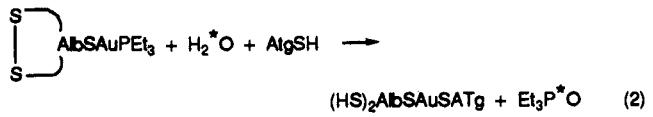
(15) Isab, A. A.; Shaw, C. F., III; Locke, J. *Inorg. Chem.* **1988**, *27*, 3406–3409.

(16) He, X.-M.; Carter, D. C. *Nature* **1992**, *358*, 209–215.

(17) Rosenoer, V. M.; Oratz, M.; Rothschild, M. A., Eds. *Albumin Structure, Function and Uses*; Pergamon Press: New York, 1977.

0002-7863/94/1516-2254$04.50/0

a rate dependent on the affinity of the thiol for gold(I).[10] The principle oxidants are disulfide bonds of the albumin, in which case the oxygen is provided by water which participates in the reaction.[15]



$$\text{AlbSAuPEt}_3 + \text{H}_2{}^*\text{O} + \text{AtgSH} \longrightarrow (\text{HS})_2\text{AlbSAuSATg} + \text{Et}_3\text{P}^*\text{O} \quad (2)$$

When chloride, a very low-affinity ligand for gold(I), is substituted for the acetylthioglucose ligand, no Et_3PO is formed.[10] If the trimethylphosphine analogue of auranofin ($Me_3PAuSATg$) is used, Me_3PO formation is more rapid and more extensive.[5] When Et_3PAuCN is used, Et_3PO forms rapidly and very extensively because the reaction is driven by the unusually high affinity of cyanide ion for gold(I).[19] These observations are consistent with an equilibrium displacement of the phosphine by AtgSH, other thiols, or cyanide, followed by an irreversible oxidation of the phosphine.

To gain further insight into the mechanism, we have undertaken studies of reactions of albumin with triisopropylphosphine analogues of auranofin (*i*-Pr_3PAuX, X = Cl, SAtg) and triisopropylphosphine itself. The *i*-Pr_3P ligand has a larger cone angle (θ) than Et_3P and is more basic as measured by the Tolman electronic parameter, ν_{CO}.[20,21] Also, the fact that the triisopropylphosphine analogue has been used in structure–function testing[22,23] means that the data from the biochemical model system can be related to in vivo results. As in our previous studies, we have employed bovine serum albumin (BSA), which is highly homologous to human serum albumin, especially in its sulfhydryl and disulfide residues and, as purchased, has a higher mercaptalbumin content (SH titer).[17] ^{31}P NMR was the principal spectroscopic method used. In the course of this research we were able to detect and isolate a transient protein–phosphonium ion intermediate in the albumin-driven oxidation of the phosphine.

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), from Boehringer Mannhein Biochemicals; elemental sulfur, KCN, hydrogen peroxide, and deuterated solvents were obtained from Aldrich Chemical Co. *i*-Pr_3P was obtained from Strem Chemicals, manipulated under inert atmosphere, and checked for purity by ^{31}P NMR. Acetamide-blocked BSA (BSA-Ac) was prepared as described earlier.[15]

^{31}P NMR Measurements. $\{^1H\}^{31}P$ NMR spectra were obtained on either a Bruker WM-250 multinuclear NMR spectrometer operating at 101.3 MHz or a General Electric GN500 operating at 202.6 MHz. The chemical shifts were measured and are reported relative to trimethylphosphate (TMP, δ_P = 2.74 relative to external 85% H_3PO_4) as an internal standard. Typical acquisition parameters were 45°, 0.54-s repetition time, and 16*K* data points. The spectral windows were −30 to 110 ppm. Approximately 5000–10 000 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 the required gold complexes in deuterated solvents were added just before the spectra were obtained.

Table 1. $^{31}P\{^1H\}$ NMR Chemical Shifts[a] of *i*-Pr_3P and Its Oxidation Products and Gold(I) Complexes

	solvent			
compound	aqueous buffer[b]	albumin soln[c]	CH_3OD	DMSO-d_6
i-Pr_3P	18.0		18.0	16.5
i-Pr_3PAuCl	60.5		63.9	63.8
i-Pr_3PO	66.7		62.3	55.8
i-Pr_3PS	71.0	70.7	70.5	70.9
i-Pr_3PAuCN[d]	64.7[d]	63.7[d]	65.0[d]	65.0[d]
i-$Pr_3PAuSAtg$	66.5	66.0	66.5	66.1
$[(i\text{-}Pr_3P)_2Au]^+$	73.7	72.7	74.0	73.9
AlbSAuP*i*-Pr_3	68.5			
$(i\text{-}Pr_3PAu)_x$BSA	58.4, 58.8, 59.8			
i-$Pr_3P^+SCH_2(HSCH_2)$Alb	75.4			

[a] Chemical shifts of the ligands and inorganic complexes were measured as 10–20 mM solutions and are reported vs internal $(MeO)_3PO$ (δ_P = 2.74 vs external 85% H_3PO_4). Values for the protein–gold complexes were measured on 2–4 mM solutions prepared in situ. The values quoted are reproducible to ±0.2 ppm. [b] 50 mM NH_4HCO_3 in 1:1 D_2O:H_2O, pH 7.9; up to 5% CH_3OD or DMSO-d_6 was used to introduce nonpolar complexes. [c] Some resonances shifted slightly downfield in the presence of albumin. [d] Ligand scrambling according to eq 3 generates [Au(P*i*-$Pr_3)_2^+$] in solution.

Analyses. Gold was quantitated by flame atomic absorption spectroscopy (AAS), and albumin was quantitated by UV absorption at 278 nm (ϵ_{278} = 39 600 L/(mol·cm)). The albumin SH titer was measured using DTNB or PySSPy. The bound gold (Au_b/BSA) and SH titer of reaction products were measured after chromatographic isolation of the product over Sephadex G-50.

***i*-$Pr_3PAuSAtg$** was prepared as previously described.[22] Anal. Calcd for $C_{23}H_{40}O_9SPAu$: C, 38.84; H, 5.60; S, 4.95. Found: C, 38.05; H, 5.51; S, 5.15.

***i*-Pr_3PAuCl** was prepared as previously described.[22] Anal. Calcd for $C_9H_{21}AuClP$: C, 27.52; H, 5.35. Found: C, 27.47; H, 5.28.

***i*-Pr_3PAuCN.** To a suspension of 112 mg (502 μmol) of AuCN in 1/1 MeOH/H_2O was added a solution of 77 mg (481 μmol) of *i*-Pr_3P in 5 mL of MeOH. After being stirred for 2 h, the solution was decanted to remove unreacted AuCN and then evaporated to dryness. The residue was washed once with H_2O and then extracted with methanol. A white crystalline complex, mp 98–101 °C dec, was obtained: 145 mg (79% yield). ν_{CN} (KBr disk): 2150. Anal. Calcd for $C_{10}H_{21}AuNP$: C, 31.33; H, 5.48; N, 3.66. Found: C, 31.11; H, 5.47; N, 3.51.

***i*-Pr_3PO.** To a solution of *i*-Pr_3P (2 mL) in ethanol (5 mL) chilled to 10 °C was added carefully 1.5 mL of 30% H_2O_2. After the vigorous reaction subsided, solvent was stripped on a rotary evaporator and the viscous residue was distilled on a short-path distillation apparatus, yielding a clear liquid, bp 126–130 °C at 15 mmHg.

***i*-Pr_3PS.** *i*-Pr_3P (500 μL) was added to elemental sulfur (0.3 g) under argon and chilled to 0 °C. After 3 h the mixture was heated to sublime the product onto a water-cooled cold finger, yielding long crystals, mp 36 °C (lit.[24] mp 37 °C). Anal. Calcd for $C_9H_{21}PS$: C, 56.21; H, 11.00. Found: C, 55.89; H, 10.96). GC–MS analysis using a Hewlett-Packard 5985 GC–MS instrument determined that the M + 1 peak (CH_4 chemical ionization) was 193, as expected. Treatment of *i*-Pr_3PS with H_2O_2 completely converted it to *i*-Pr_3PO.

Reaction of BSA and *i*-Pr_3PAuCl. BSA (4.04 mM, SH titer = 0.62, 1.8 mL) was treated with successive aliquots of *i*-Pr_3PAuCl (66.7 mM in DMSO), yielding Au_i/BSA ratios of 0.73, 1.47, and 2.84. After each addition, the ^{31}P NMR spectrum was measured. No precipitates were observed after the reagents were mixed nor after the spectral accumulations were completed (~6 h). After the first accumulation, an aliquot was set aside for later chromatographic analysis to determine the Au_b/BSA ratio, 0.65. Aliquots of a freshly prepared ATgSH solution (100 mM in methanol) were added successively to the last solution (Au_i/BSA = 2.84) to give Au/BSA/ATgSH ratios of 2.84/1/1.37 and 2.84/1/4.12. ^{31}P NMR spectra were obtained after each addition. An aliquot of the final solution was chromatographed to determine the Au_b/BSA, 0.97 (see Table 2).

Reaction of BSA and *i*-$Pr_3PAuSAtg$. BSA (4.04 mM, SH titer = 0.62, 1.8 mL) was mixed with *i*-$Pr_3PAuSAtg$ (86 mM in MeOH) giving

(18) Ecker, D. J.; Hempel, J. C.; Sutton, B. M.; Kirsch, R.; Crooke, S. T. *Inorg. Chem.* **1986**, *25*, 3139–3143.
(19) Isab, A. A.; Hormann-Arendt, A. L.; Coffer, M. T.; Shaw, C. F., III *J. Am. Chem. Soc.* **1988**, *110*, 3278–3284.
(20) Tolman, C. *Chem. Rev.* **1977**, *77*, 313–345.
(21) McAuliffe, C. A. In *Comprehensive Coordination Chemistry*; Wilkinson, G., Ed.; Pergamon Press: New York, 1987; Vol. 2, Chapter 14, pp 989–1066.
(22) Hoeschele, J. D. *Proceedings of the International Symposium on the Synthesis and Application of Isotopically Labelled Compounds*; June 6–11, 1982; Duncan, W. P., Susan, A. B., Eds.; Elsevier: Amsterdam, 1983; pp 353–354.
(23) Sutton, B. M.; McGusty, E.; Walz, D. T.; DiMartino, M. J. *J. Med. Chem.* **1972**, *15*, 1095–1098.
(24) Tiethof, J. A.; Hetey, A. T.; Meek, D. W. *Inorg. Chem.* **1974**, *13*, 2505–2509.

Table 2. Gold to Albumin Binding Ratios for i-Pr_3PAuX (X = Cl,AtgS) with and without Competing Ligands

reactants[a]						product[b]
X	[*i*-Pr_3PAuX]	[BSA]	SH titer	ligand,[c] concn	Au_i/BSA	Au_b/BSA
Cl	2.67	3.88	0.62		0.69	0.65
Cl	2.45	3.56	0.62	CN^-,0.32	0.69	0.32
Cl	8.58	3.02	0.62	AtgSH,12.45	2.84	0.97
AtgS			0.66		1.18	0.50
AtgS	3.64	3.08		CN^-,11.8	1.18	0.24

[a] Concentrations are in millimolar. [b] Au_b/BSA was determined after the reaction mixture was fractionated over a Sephadex G-50 resin. [c] Competing ligands were added to the albumin adducts and incubated at least 1 h before they were fractionated on a gel-exclusion resin.

Au_i/BSA = 0.69. A ^{31}P NMR spectrum was obtained, and an aliquot was set aside for analysis of Au_b/BSA. Into the remaining reaction mixture, aliquots of 0.1 M KCN in 0.1 M NH_4HCO_3 buffer were added, yielding CN/Au_i ratios of 0.31, 1.23 and 3.29; spectra were accumulated after each addition.

Reaction of BSA and *i*-Pr_3P. *i*-Pr_3P (3 μmol in 60 μL of MeOH) was added under inert atmosphere to a buffered solution of BSA (2.0 mM, 1.5 mL in 100 mM NH_4HCO_3, pH 7.9) and to control samples containing only buffer. ^{31}P NMR spectra were accumulated at 202.6 MHz in 1-h data blocks for 18–24 h after mixing. The integrated intensities of the 75.4-ppm resonance assigned to *i*-$Pr_3P^+SCH_2(HSCH_2)$Alb were used to calculate the rate of its disappearance.

In some cases the reactions were carried out with iodoacetamide-blocked albumin (BSA-Ac) in which Cys-34 had been converted to a thioether so that the thiols generated by the *i*-Pr_3P could be analyzed without interference by Cys-34. Aliquots of reactions mixtures similar to those for the NMR studies were fractionated over Sephadex G-25 to remove any unreacted phosphine, and the protein fractions were analyzed using dithiopyridine (PySSPy) to determine the SH titer.

Results

The ^{31}P NMR chemical shifts of triisopropylphosphine, its oxide, its sulfide, and the gold complexes used here were measured in aqueous solutions prepared by adding small volumes of concentrated methanolic or DMSO solutions of the compounds to 100 mM NH_4HCO_3 buffer, pH 7.9. In some cases, solubility was a problem, but for every compound a spectrum was obtained. The NMR shifts in aqueous NH_4HCO_3 buffer, CH_3OD, and DMSO-d_6 are reported in Table 1. For the chloro-, tetraacetylthioglucose-, and bis(phosphine)gold(I) complexes, only single resonances were observed. For the cyano complex, however, two resonances were present in aqueous solution: 64.7 ppm, assigned to *i*-Pr_3PAuCN, and 73.7 ppm, assigned to [(*i*-$Pr_3P)_2Au^+$]. The latter arises via the ligand-scrambling (redistribution) reaction of eq 3:[14,25]

$$2i\text{-}Pr_3PAuCN \rightleftharpoons (i\text{-}Pr_3P)_2Au^+ + Au(CN)_2^- \quad (3)$$

The extent of the scrambling reaction, as indicated by the relative intensities of the NMR resonances, was greatest in the aqueous medium, which is more polar than methanol or DMSO, as expected for an equilibrium between a neutral complex and ionic derivatives.

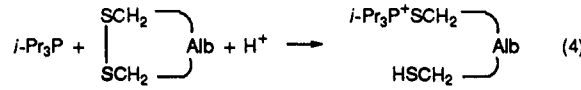
i-Pr_3PO and *i*-Pr_3PS were prepared from *i*-Pr_3P so that their spectra could be obtained in aqueous solution. The chemical shift of *i*-Pr_3PO, generated by H_2O_2 or by autooxidation, was 64.3 ppm in buffer. For *i*-Pr_3PS, generated by reaction with elemental sulfur, the shift was 71 ppm in 50% MeOH/D_2O. The sulfide was also subjected to GC–MS analysis, using chemical ionization, to establish its identity, $m + 1/e = 193$. Reaction with H_2O_2 converted *i*-Pr_3PS into *i*-Pr_3PO; conversion could also be effected by allowing the 1/1 MeOH/D_2O solutions to stand for 1 week at room temperature.

(25) Hormann-Arendt, A. L.; Shaw, C. F., III *Inorg. Chem.* **1990**, *29*, 4683–4687.

During the course of the studies described below, it was observed that several of these complexes undergo a systematic shift of –0.3 to –1.0 ppm in the presence of serum albumin. The shifts observed with 1–2 mM compounds in the presence of 1–2 mM albumin in the ammonium carbonate buffer are also listed in Table 1.

***i*-Pr_3P Reactions with Albumin.** The observation of an unusual resonance at 75.4 ppm in certain albumin reactions discussed below prompted us to examine the direct reaction of *i*-Pr_3P with the protein. One equivalent of *i*-Pr_3P was added to an albumin solution, and spectra were accumulated at 1-h intervals. The 75.4-ppm resonance (Figure 4) appeared immediately after mixing and progressively decreased over 14 h. The 70.7-ppm resonance of *i*-Pr_3S grew in over this period. There was only a slight increase in the amount of *i*-Pr_3PO (δ_P = 66.5), which is also present as an impurity in the *i*-Pr_3P. The small starred peak at 61.3 ppm is an impurity from the commercial phosphine preparation that did not change intensity during the reaction. Under scrupulously anaerobic conditions, control spectra of *i*-Pr_3P in the buffer solution showed primarily *i*-Pr_3P (18.0 ppm) with traces of *i*-Pr_3PO and the 61.3-ppm impurity, while aerobically mainly *i*-Pr_3PO is observed.

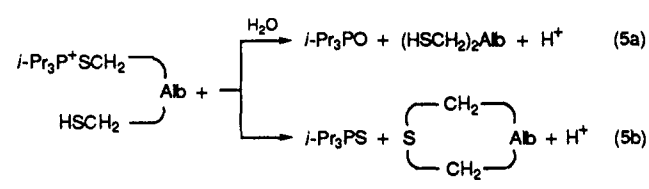
Serum albumin contains 17 disulfide bonds, generated from 34 of its 35 cysteine residues and a fractional population of mixed disulfides of cysteine and glutathione formed at Cys-34. The reduction of a disulfide by a phosphine in polar media occurs via a phosphonium ion intermediate with a thiolate ligand, $R_3PSR'^+$.[26] Therefore, we tentatively assigned the 75.4-ppm resonance to a protein phosphonium derivative in which -SR is -SCH_2-($HSCH_2$)-Alb, a cysteine side chain that is generated by cleaving a disulfide and is covalently bound to the isopropylphosphonium center:



(4)

In several instances, the protein was chromatographically fractionated over a gel-exclusion resin immediately after the phosphine was added to the solution. After reconcentration of the protein, ^{31}P NMR spectra were obtained. The presence of the 75.4-ppm resonance in these spectra (not shown) confirms our assignment of the peak to a protein-bound intermediate. The low molecular weight species present elute after the protein and cannot be the source of this resonance.

The well-documented chemistry of the reactions of disulfides with trivalent phosphines[26] indicates that *i*-$Pr_3P^+SCH_2(HSCH_2)$-Alb should be irreversibly converted to the phosphine sulfide and/or oxide species. The oxide forms via nucleophilic attack of water on the phosphonium species with displacement of the thiolate (eq 5a). The sulfide can be liberated via attack of the free thiol group at the methylene carbon on the phosphonium thiolate, generating a monosulfide (i.e., a thioether) in place of the original disulfide bond (eq 5b):



The formation of *i*-Pr_3PS (δ_P = 71 in Figure 1) indicates that the second pathway dominates in this reaction.

Pathway 5a predicts an increase in the albumin SH titer as the phosphonium ion of *i*-$Pr_3P^+SCH_2(HSCH_2)$ Alb is hydrolyzed, while pathway 5b predicts a decrease as the free thiol displaces *i*-Pr_3PS. Therefore, the SH titer of iodoacetamide-blocked

(26) Mukaiyama, T.; Takei, H. *Top. Phosphorous Chem.* **1976**, *8*, 587–646.

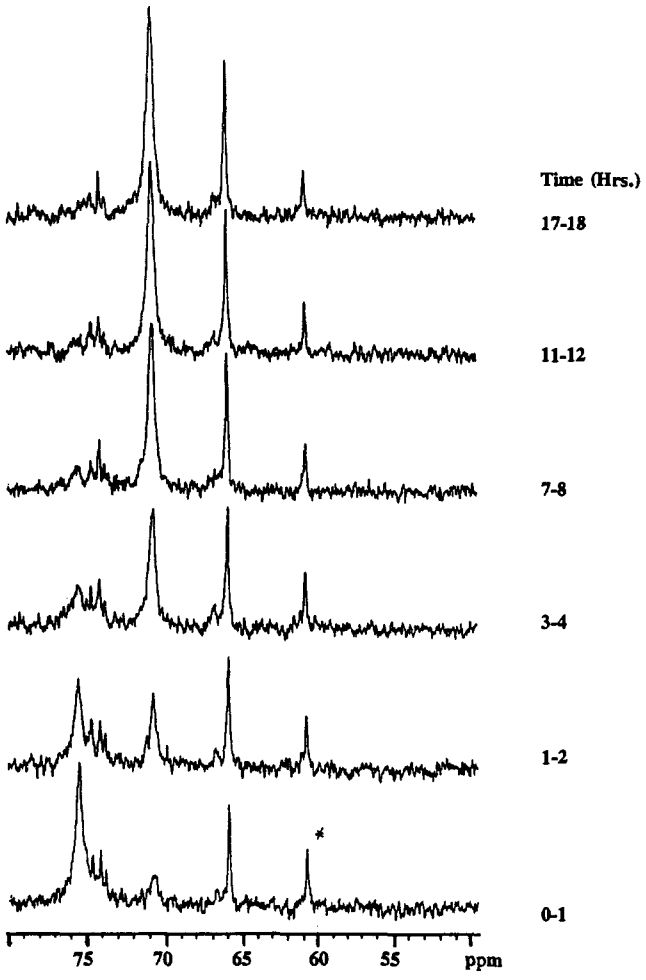


Figure 1. $\{^1H\}^{31}P$ NMR spectra (202.6 Hz) of serum albumin (2.0 mM) after reaction with 1 equiv of i-Pr_3P in buffered aqueous solution (100 mM NH_4HCO_3, pH 7.9). Spectra were accumulated in 1-h segments over an 18-h period, and representative time points are shown. The signal at 75.4 ppm attributed to i-$Pr_3P^+SCH_2(HSCH_2)$BSA appears immediately and decays with concomitant formation of i-Pr_3PS (70.1 ppm) and i-Pr_3PO (66.7 ppm). The signal starred in the bottom spectrum is an impurity observed along with i-Pr_3PO in a spectrum of i-Pr_3P in buffer/methanol solution under strictly anaerobic conditions.

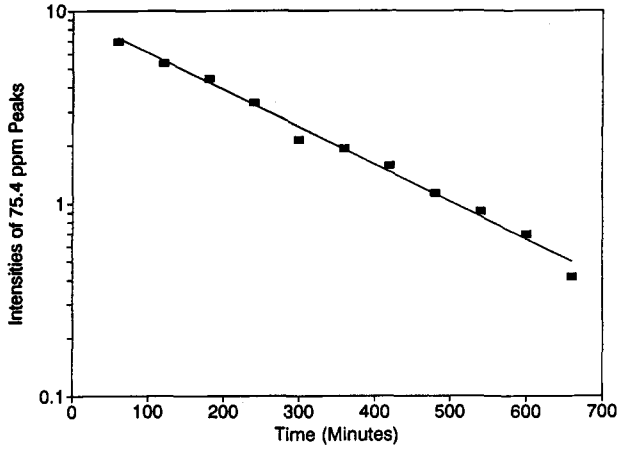


Figure 2. Kinetic analysis of the intensity of the i-$Pr_3P^+SCH_2(HSCH_2)$-Alb resonance at 75.4 ppm. T = 25 °C; other conditions as in Figure 1. The linear slope indicates a first-order loss of i-$Pr_3P^+SCH_2(HSCH_2)$-Alb, with $k_{obs} = (6.9 \pm 0.6) \times 10^{-5}$ s^{-1}.

albumin (BSA-Ac) after reaction with i-Pr_3P under anaerobic conditions was examined over time (using PySSPy) to distinguish between the reaction pathways. Using BSA-Ac (in which the free thiol, Cys-34, of mercaptalbumin, Alb-SH, is converted to

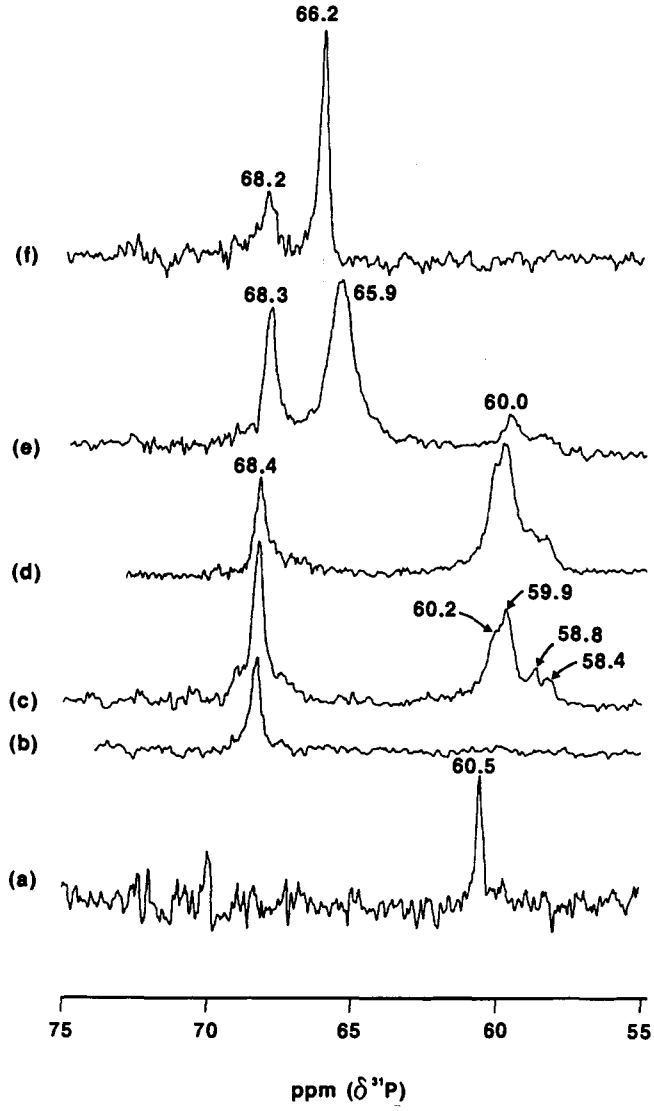


Figure 3. $\{^1H\}^{31}P$ NMR spectra of albumin–gold complexes. (a) 0.75 mM i-Pr_3PAuCl; (b–d) 4.04 mM BSA in buffered aqueous solution, pH 7.9, treated with successive additions of 66.7 mM i-Pr_3PAuCl to yield the concentrations of BSA and Au given below; (e,f) aliquots of 100 mM AtgSH were added successively to the sample. Concentrations (mM) of [Au]:[BSA]:[AtgSH]: (a) 0.75:0:0; (b) 2.84:3.87:0; (c) 5.44:3.71:0; (d) 9.80:3.45:0; (e) 9.36:3.29:4.52; (f) 8.58:3.02:12.5.

$AlbSCH_2CONH_2$) eliminates complications due to preexisting cysteine in the protein. The 17 inernal disulfides and the mixed disulfides at Cys-34 are known to react with reductants such as phosphines and thiols.[27,28] The NMR experiments above demonstrated that free i-Pr_3P is not present and, therefore, cannot react directly with the PySSPy reagent used to quantitate the protein thiols. Measurements carried out on aliquots of the reaction mixtures yielded SH titers of 0.6–0.7 shortly after BSA-Ac and i-Pr_3P were mixed and showed a progressive decrease of SH titer over time. This result is consistent with pathway 5b and the finding that i-Pr_3PS is the reaction product. It also supports the assignment of the 75.4-ppm resonance to i-$Pr_3P^+SCH_2$-$(HSCH_2)$Alb.

The intensity of the resonance at 75.4 ppm is lost over 14–18 hours at 25 °C as the NMR spectra are accumulated, Figure 1. When the integrated intensity of the peak is plotted on a semilogarithmic scale, the resulting plot is linear, indicative of a first-order reaction, Figure 2. The observed rate constant, k_{obs},

(27) Isab, A. A.; Hormann-Arendt, A. L.; Hill, D. T.; Griswold, D. E.; DiMartino, M. J.; Shaw, C. F., III *Inorg. Chem.* **1989**, *28*, 1321–1326.
(28) Ni Dhubhughaill, O. M.; Sadler, P. J.; Tucker, A. *J. Am. Chem. Soc.* **1992**, *114*, 1118–1120.

is $(6.9 \pm 0.6) \times 10^{-5}$ s^{-1}. The reaction of a single substrate via two independent pathways (e.g., eqs 5a and 5b) yields a single exponential with a rate constant that is the sum of the two paths, providing that both are first-order or pseudo-first-order.[29] The formation of i-Pr_3PS should be first-order in protein since it is an internal rearrangement, and the hydrolysis should be pseudo-first-order since H_2O is present in large excess. Thus, designating the rate constants for the pathways of 5a and 5b as $k_{P=S}$ and $k_{P=O}$, the relationship

$$k_{obs} = k_{P=S} + k_{P=O}$$

applies to this reaction. The NMR data show that the formation of i-Pr_3PS dominates and, hence, that $k_{P=S} \approx k_{obs}$ and that $k_{P=O}$ is significantly smaller.

Albumin Reactions with i-Pr_3PAuX. Examination of the reactions of the isopropylphosphinegold(I) complexes with bovine serum albumin was initiated by studying the reaction of i-Pr_3-PAuCl with a serum albumin sample having a SH titer (i.e., mercaptalbumin mole fraction) of 0.62. When i-Pr_3PAuCl dissolved in DMSO-d_6 and sufficient to be equimolar with the mercaptalbumin component (AlbSH) was added to a buffered solution, the 60.5-ppm resonance of the i-Pr_3PAuCl (shown in Figure 3a) was absent, and a new resonance at 68.5 ppm appeared (Figure 3b) and was tentatively assigned as AlbSAuPi-Pr_3. To verify this assignment, i-$Pr_3PAuSAtg$ (Au_i/BSA = SH titer) was reacted with a similar albumin solution. A resonance at 68.5 ppm (not shown) appeared and can be attributed to AlbSAuPi-Pr_3. The formation of the resonance at 68.5 ppm using two complexes differing in the anionic ligand is consistent with ligand-exchange reactions at gold(I), according to eqs 6 and 7:

$$\text{AlbSH} + i\text{-Pr}_3\text{PAuCl} \rightleftharpoons \text{AlbSAuP}i\text{-Pr}_3 + \text{H}^+ + \text{Cl}^- \quad (6)$$

$$\text{AlbSH} + i\text{-Pr}_3\text{PAuSAtg} \rightleftharpoons \text{AlbSAuP}i\text{-Pr}_3 + \text{AtgSH} \quad (7)$$

Previous studies have identified the cysteine-34 residue of mercaptalbumin as the tight binding site for gold(I) species.[4-9] The downfield shift of the phosphorus resonance when acetylthioglucose or chloride is displaced R_3PAuX by Cys-34 has been observed when R_3P is Me_3P or Et_3P.[4,5,7,8,10]

When i-$Pr_3PAuSAtg$ in excess of that required to saturate the mercaptalbumin component was added to albumin, only the signals for AlbSAuPi-Pr_3 and the complex itself were observed. Thus, histidine and other potential weak binding sites are unable to displace the AtgSH from the gold(I). In contrast, when similar excesses of i-Pr_3PAuCl were added to albumin, the ^{31}P NMR spectra contained additional, overlapping resonances at 58.4, 58.8, 59.9, and 60.2 ppm (Figure 3c,d). The three at higher fields resemble the broad resonances observed in reactions of Me_3-PAuCl and Et_3PAuCl with albumin[4,5] and are assigned as i-Pr_3-PAu^+ at weak binding sites, presumed to be histidine (and possibly methionine) residues, and, thus, are assigned as $(i\text{-Pr}_3\text{PAu})_x$-AlbSAuP$i$-$Pr_3$. The resonance at 60.2 ppm may arise from either an additional weak binding site for i-PrP_3Au^+ or, since the chemical shift is close to that of i-Pr_3PAuCl (Table 1) and the signal is broadened, i-Pr_3PAuCl bound at one of the hydrophobic binding sites on albumin.

To verify that the resonances between 58 and 61 ppm represent i-Pr_3PAu^+ bound weakly and reversibly, acetylthioglucose (AtgSH), which binds gold(I) more strongly than histidine or thioether residues, was added to the solution. Addition of about 0.5 equiv (based on gold) reduced the intensity of the weak binding sites and generated a broad peak at 65.9 ppm (Figure 3e). A second aliquot (1.5 equiv total of AtgSH) removed most of the gold from the weak binding sites and caused the broad peak to shift to 66.2 ppm and sharpen (Figure 3f). This resonance falls between the chemical shifts observed for free i-$Pr_3PAuSAtg$ in buffer, 66.5 ppm, and in the presence of albumin, 66.0 ppm (Table 1). Thus, the 65.9-ppm resonance is assigned to i-$Pr_3PAuSAtg$ in exchange with the weak binding sites, consistent with its broadness and intermediate position, and the 66.2-ppm resonance to free i-Pr_3-PAuSAtg. The loss of the weakly bound gold and even some of the gold at the strong binding site, Cys-34, confirms the equilibrium nature of the reactions. The relative affinities of chloride, tetraacetylthioglucose, and the albumin Cys-34 and weak binding sites for triisopropyl phosphinegold(I) are AlbSH > AtgSH > weak binding sites > Cl^-. Thus, substituting the bulky and more basic triisopropylphosphine for triethylphosphine in R_3PAu^+ does not change the ordering found with R = Me and Et.[4,7,8]

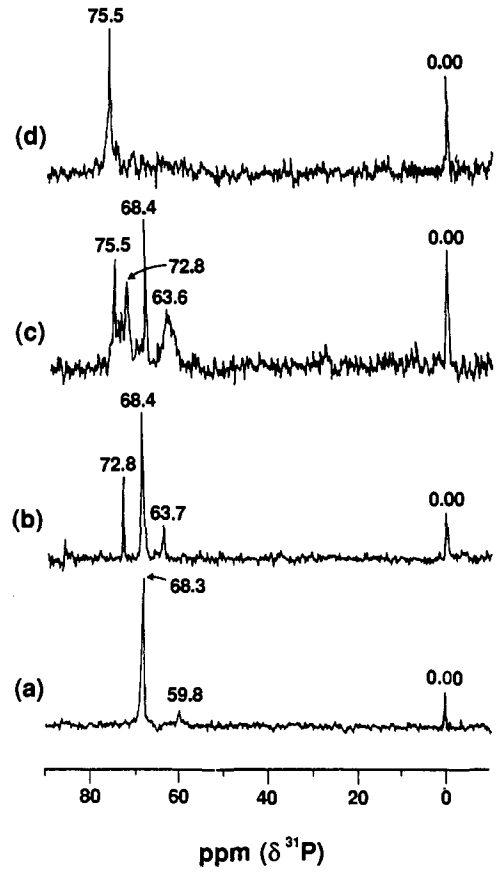


Figure 4. $\{^1H\}^{31}P$ NMR spectra of albumin–gold complexes after reaction with cyanide. BSA with an SH titer of 0.62 was treated with i-Pr_3PAuCl (Au_i/BSA = 0.68) to which increasing ratios of KCN were added. Concentrations (mM) of [BSA]:[Au]:[CN]: (a) 3.88:2.67:0; (b) 3.85: 2.65:0.82; (c) 3.76:2.59:3.18; (d) 3.56:2.45:8.06.

The absence of the signals at 75.4 ppm for the phosphonium intermediate and at 71 ppm for i-Pr_3PS when AtgSH reacts with the albumin–gold–phosphine complex suggests that i-Pr_3P is not being displaced by AtgSH. Long-term experiments (not shown) verified that even over 24 h (in which time 30–40% of the Et_3P from $AlbSAuPEt_3$ is displaced and oxidized to Et_3PO[10]), no i-$Pr_3P^+SCH_2(HSCH_2)$Alb or i-Pr_3PO and only a trace of i-Pr_3-PS was detected. A second set of experiments was conducted using AlbSAuPi-Pr_3 and penicillamine (PaSH), which is also a high-affinity ligand for gold(I),[30] to test for displacement of the i-Pr_3P. After 15 h, less <1% of the i-Pr_3P had been converted to i-Pr_3PS. Therefore, it can be concluded that i-Pr_3P is displaced from its albumin-bound gold(I) complex much less readily than either Et_3P or Me_3P from the analogous complexes.

Recently, cyanide has been shown to play an important role in the in vivo chemistry of gold complexes in cigarette smokers[31]

(29) Espenson, J. *Chemical Kinetics and Reaction Mechanisms*; McGraw Hill: New York, 1981; pp 55–56.

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

(31) Graham, G. G.; Haavisto, T. M.; Jones, H. M.; Champion, G. D. *Biochem. Pharmacol.* **1984**, *33*, 1257–1262.

and nonsmokers.[32] Therefore, we examined the reaction of the AlbSAuP*i*-Pr_3 complex with HCN (generated by the hydrolysis of KCN, which is nearly complete (~95%) at pH 7.9). Figure 4 shows the sequence of spectra obtained as the cyanide was progressively added to a sample of AlbSAuP*i*-Pr_3 generated in situ by reaction of albumin (SH titer = 0.66) with 1 equiv (based on AlbSH) of *i*-Pr_3PAuCl. Before the addition of cyanide (Figure 4a), only *i*-Pr_3PAuSAlb and a trace of weakly bound *i*-Pr_3PAu^+ were present. After the addition of 0.3 equiv of cyanide (Figure 4b), the resonances of *i*-Pr_3PAuCN at 63.8 ppm and [(*i*-$Pr_3P)_2Au^+$] at 72.8 ppm appeared and then increased in intensity as additional cyanide (0.6 equiv total) was added. [(*i*-$Pr_3P)_2Au^+$] forms from *i*-Pr_3PAuCN via the ligand-scrambling reaction of eq 3. After chromatography, recovery, and reconcentration of an aliquot of the albumin, only the residual AlbSAuP*i*-Pr_3 resonance was observed, confirming that the 72.8- and 63.8-ppm resonances are not due to protein-bound species. The displacement and subsequent scrambling reactions occur as follows:

$$\text{AlbSAuP}i\text{-Pr}_3 + \text{HCN} \rightleftharpoons \text{AlbSH} + i\text{-Pr}_3\text{PAuCN} \rightleftharpoons (i\text{-Pr}_3\text{P})_2\text{Au}^+ + \text{Au(CN)}_2^+ \quad (8)$$

After a total of 1.2 equiv (per gold) of cyanide were added, the resonance of *i*-$Pr_3P^+SCH_2(HSCH_2)$Alb appeared at 75.4 ppm (Figure 4c). This species was identified and characterized in the direct reactions of *i*-Pr_3P and albumin described above. After a large excess (3.3 equiv) had been added, only the 75.4-ppm resonance was observed (Figure 4d). Over 24 h, this band at 75.4 ppm disappeared and was replaced by resonances for *i*-Pr_3PO and *i*-Pr_3PS at 64.3 and 71.0 ppm, respectively (not shown).

i-$Pr_3P^+SCH_2(HSCH_2)$Alb (75.4 ppm) was not observed in any of the direct reactions of *i*-Pr_3PAuCl or *i*-Pr_3PAuSAtg with HCN described below but is observed in the reaction of *i*-Pr_3P with albumin described above. Its formation during the cyanide titration of AlbSAuP*i*-Pr_3 in Figure 4 can be explained as follows. When the cyanide concentration exceeds that of gold, *i*-Pr_3P is displaced from the gold(I) (eq 9a). The free phosphine is not observed in Figures 2c, 2d, or 3 because it rapidly reacts with an albumin disulfide bond to form the phosphonium intermediate and a reduced thiol according to eq 9b (or eq 4 above).

$$i\text{-Pr}_3\text{PAuCN} + \text{HCN} \rightleftharpoons i\text{-Pr}_3\text{P} + \text{H}^+ + \text{Au(CN)}_2^- \quad (9a)$$

$$i\text{-Pr}_3\text{P} \xrightarrow[\text{fast}]{\text{albumin}} i\text{-Pr}_3\text{PS}^+\text{CH}_2(\text{HSCH}_2)\text{Alb} \quad (9b)$$

Model Reactions of *i*-Pr_3PAuX with Cyanide. In contrast to the reaction of HCN with AlbSAuP*i*-Pr_3, reactions of *i*-Pr_3PAuSAtg and cyanide should not produce the phosphine oxide or sulfide if, indeed, albumin disulfide bonds are the oxidant and sulfur source. Therefore, inorganic model reactions were studied in DMSO-d_6. When <1 equiv of cyanide was added to *i*-Pr_3PAuSAtg, the products and reactants were in a fast-exchange regime and only a single broad resonance was observed. When cyanide was added to *i*-Pr_3PAuCl, resonances of *i*-Pr_3PAuCl (64.0 ppm), *i*-Pr_3PAuCN (65.0 ppm), and (*i*-$Pr_3P)_2Au^+$ (73.9 ppm) were identified in the spectrum. The cyanide arises by ligand exchange, eq 10,

$$i\text{-PrAuCl} + \text{HCN} \rightarrow i\text{-Pr}_3\text{PAuCN} + \text{Cl}^- + \text{H}^+ \quad (10)$$

and the bisphosphine cation by the ligand-scrambling reaction, eq 3.

When excess cyanide was added, *i*-Pr_3P was observed (eq 11). No resonances for *i*-Pr_3PO, *i*-Pr_3PS, or *i*-$Pr_3P^+SCH_2(HSCH_2)$-Alb were observed in any of the spectra. The observation of free

$$i\text{-Pr}_3\text{PAuX} + \text{KCN(excess)} \xrightarrow{\text{DMSO}} i\text{-Pr}_3\text{P} + \text{Au(CN)}_2^- + 2\text{K}^+ \quad (11)$$

i-Pr_3P and the absence of the 75.4-ppm resonance in these protein-free model reactions and, conversely, the absence of *i*-Pr_3P and the formation of the 75.4-ppm resonance in the presence of albumin are consistent with the assignment of the latter as *i*-Pr_3P^+-$SCH_2(HSCH_2)$Alb.

Discussion

The chemical basis for the metabolism of metallodrugs is intrinsically different than that for organic drugs. The latter generally involve functional group reactions that leave the carbon skeleton intact, while metallodrugs undergo ligand-exchange and metal-centered redox reactions. Gold-based antiarthritic drugs provide a special challenge, since gold(I) is extremely labile to ligand-exchange reactions and the ligands and metal ion may have independent metabolic fates, Understanding the nature of the metabolites formed and the reaction mechanisms which generate them is crucial to future advances in unraveling the mechanism(s) of action of chrysotherapy and designing the third generation of gold drugs.

The goal of these studies is to elucidate the chemical reactions and mechanisms which underly auranofin metabolism. Facile accumulation of the ^{31}P NMR spectra necessarily required the use of millimolar concentrations of albumin and the gold complexes, which are respectively 2–5-fold and somewhat more than 2 orders of magnitude greater than the relevant in vivo concentrations. The rationale for doing so is that the stoichiometry of the reactions and the nature (structure) of the products and intermediates formed are concentration independent. In contrast, application of our findings to analysis of detailed pharmacokinetics requires careful adjustment for the differences in the relative and absolute concentrations of gold and albumin found in vivo. Nonetheless, our qualitative findings (e.g., the structure–function relationship for phosphine oxidation (*i*-$Pr_3 < Et_3P < Me_3P$)) and the quantitative findings (e.g., the first-order rate constant for *i*-$Pr_3P^+SCH_2(HSCH_2)$Alb decay) provide a firm chemical basis for further research on the medicinal chemistry and molecular pharmacology of antiarthritic gold compounds.

The isolation and ^{31}P NMR characterization of *i*-$Pr_3P^+SCH_2$-$(HSCH_2)$Alb, δ_P = 75.4, provide the first spectroscopic evidence for an albumin–phosphonium intermediate in the oxidation of trialkylphosphines and provide strong support for the previously proposed mechanism of phosphine displacement and subsequent oxidation.[10,15] Previous evidence for this reaction includes the increase of the albumin SH titer when Et_3PO is generated in AlbSAuPEt_3,[15] the incorporation of ^{17}O from $H_2{}^{17}O$ into Et_3-PO,[15] the similarity of the rate of formation of Et_3PO under anaerobic and aerobic conditions,[10] and the denaturation of albumin by excess [$Au(PEt_3)_2^+$][7]. The formation of *i*-Pr_3PS in this case provides further direct evidence for the role of the disulfide bond as an oxidant.

The course of the AlbSAuP*i*-Pr_3 and AlbSAuPEt_3 reactions with cyanide are quite different from one another. In each case, the cyanide rapidly liberates a free phosphine which reacts with an albumin disulfide bond. However, the triethylphosphine derivative generates Et_3PO very rapidly,[19] while the triisopropyl derivative generates *i*-$Pr_3P^+SCH_2(HSCH_2)$Alb, which reacts slowly to form *i*-Pr_3PS. That is, Et_3P reacts according to a pathway analogous to eq 5a, while *i*-Pr_3P follows the pathway of eq 5b. These differences in the reaction mechanisms and resulting

(32) Elder, R. C.; Shao, Z.; Shang, Y.; Korsey, J. G.; Hess, E. B.; Tepperman, K. G. *J. Rheumatol.* **1993**, *20*, 268–272.

products can be attributed to the steric and electronic influences of the isopropyl groups. Attack of water at the phosphine is required for formation of R_3PO, while sulfur attack at the β-carbon of the phosphonium thiolate group leads to R_3PS formation. The bulky isopropyl groups apparently hinder access of water to the phosphorus atom, thereby allowing the slower pathway leading to sulfide to predominate.

Equally significant is the finding that AtgSH does not displace the isopropylphosphine from AlbSAuP*i*-Pr_3 to generate *i*-Pr_3P^+-$SCH_2(HSCH_2)$Alb and ultimately *i*-Pr_3PO or *i*-Pr_3PS. In contrast, AtgSH and AlbSAuPEt_3 react slowly to generate Et_3PO.[10] These findings, combined with earlier results for AlbSAuPMe_3,[5] allow us to establish a structure–function relationship for the ease of displacement of trialkylphospines from their albumin–gold complexes: $Me_3P > Et_3P >$ *i*-Pr_3P. This order can be compared to the Tolman cone angles (θ = 118°, 132°, and 160°)[20] and electronic factor (ν = 2064.1, 2061.7, and 2059.2, respectively).[20] Increasing basicity of the phosphine (decreasing value of ν) and increasing steric hindrance (increasing θ) should both contribute to a less favorable transition state for phosphine displacement across the series.

The empirical observation that *i*-Pr_3P is not readily displaced has important implications for the design of third-generation chrysotherapy drugs. Use of higher affinity phosphines, which are not displaced readily, may allow the design of gold drugs targeted to specific cells which are critical to etiology of rheumatoid arthritis. This might be accomplished using modified phosphines, R_s*i*-Pr_2P, in which a substituted alkyl or aryl group, R_s, includes substituents which favorably alter the in vivo distribution of the gold(I) during the interval preceding phosphine displacement and oxidation.

Acknowledgment. This research was supported by NIH (AR 39902) and has benefited from NMR instrumentation supported by the Division of Research resources of the NIH (RR 04095) and the Biological and Chemical Instrumentation Programs of the NSF (DIR-8719808). A.A.I. thanks The King Fahd University of Petroleum and Minerals for a sabbatical leave during the 1986–87 academic year. C.F.S. thanks the UWM Graduate School for matching funds for purchasing a UV–vis spectrometer and for a research assistantship for J.X. We thank Mr. Frank Laib for obtaining the GC–MS spectra.