

A ^{31}P Nuclear Magnetic Resonance and Fluorescence Study of the Interaction of an Anti-arthritic Gold Phosphine Drug with Albumin. A Bioinorganic Approach

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^1H decoupled ^{31}P nmr spectra were recorded for a series of gold complexes of formulae PEt_3AuL and $[\text{PEt}_3\text{AuL}]^+\text{ClO}_4^-$ where L and L' are ligands containing biologically relevant donor atoms. This series of a model compounds provide a ^{31}P nmr scale for the interaction of the ' PEt_3Au ' moiety with proteins. The reactions of albumin and SH blocked albumin with PEt_3AuCl were monitored by ^{31}P nmr spectroscopy. Comparison of the observed chemical shifts to those of the model compounds revealed preferential binding of gold to S occurs. Fluorescence studies of the gold–protein interactions imply that a protein conformational change occurs on binding of gold. The implications of these studies on the mechanism of action of anti-arthritic gold drugs is discussed.

Introduction

Recent reviews of gold chemistry have focused on applications in biology and medicine [1–7]. Of particular interest has been the use of gold thiolates in the treatment of rheumatoid arthritis [1, 4, 7]. Side effects with these drugs can be severe, nonetheless, the ability of chrysotherapy (gold treatments) to cause remission of the disease results in these drugs being one of the standard prescriptions of modern medicine for severe cases of rheumatoid arthritis. The past decade has seen significant progress in reducing adverse side effects by the introduction of new gold–phosphine drugs [4, 8, 9]. These drugs are readily absorbed through the gut; thus they have the additional benefit of oral administration, in lieu of the painful intramuscular injections that are required with the gold–thiolate drugs.

The mechanism of action of gold drugs has been the subject of speculation. Generally many of the proposals deal with the alteration of enzyme function by gold complexation, however the exact enzyme system or systems involved are unknown [2]. Initial *in vivo* studies investigated gold distributions throughout the body following drug administration [7]. A

large proportion (50–90%) of the gold is present in the blood bound to the serum protein, albumin. Despite its apparent affinity for gold, little is known regarding the nature or effects of gold–phosphine drug binding to albumin.

We investigated the interactions of gold–phosphine drugs and albumin employing a bioinorganic approach [10]. First, we examined by ^{31}P nmr spectroscopy an extensive series of model compounds designed to mimic the interactions of the ' PEt_3Au ' moiety with biologically relevant donor ligands. Secondly ^{31}P nmr studies of the reactions of PEt_3AuCl with albumin were undertaken. This two phase approach provided a basis for conclusions regarding the nature of the gold binding to the protein. Effects of gold binding on protein conformation were investigated by fluorescence spectroscopy. The results of these studies are the subject of this paper.

Experimental

Preparations of all gold compounds were performed under an atmosphere of dry, O_2 -free N_2 . ^{31}P nmr spectra were recorded at 25°C on a Bruker CXP-100 pulse nmr spectrometer operating at 36.4 MHz with broad band proton decoupling. The ^{31}P chemical shifts are reported relative to 85% H_3PO_4 . All fluorescence data were recorded on locally assembled fluorometer consisting of an Oriole Xenon lamp, McPherson emission monochromator and a Heath exciting monochromator. All ligands were purchased from the Aldrich Chemical Co. or Sigma Chemicals. HAuCl_4 was obtained on loan from Johnson-Matthey Ltd. Human serum albumin (HSA) and bovine serum albumin (BSA) were purchased from Sigma Chemicals.

Preparation of Gold Compounds

PEt_3AuCl was prepared from HAuCl_4 according to literature methods [11]. $\text{PEt}_3\text{AuCl}_3$ and $(\text{PEt}_3\text{Au})_2\text{S}$ were prepared by known routes [12, 13].

Preparation of PEt_3AuL

All compounds 2–18 were prepared using methods analogous to those published in the literature

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[13–15]. In some cases NBu_4OH (1 *M* in methanol) was used in lieu of NaOCH_3 as the base. A sample preparation is given below.

50 mg (0.143 mmol) of PEt_3AuCl was dissolved in methanol (10 ml). Separately 15.7 mg of $\text{C}_6\text{H}_5\text{SH}$ is added to one equivalent of NaOCH_3 solution. The two solutions are combined and stirred for 1 h. The solvent is removed, the residue dissolved in CDCl_3 and NaCl is removed by filtration through celite. The CDCl_3 contained pure $\text{PEt}_3\text{Au-SC}_6\text{H}_5$ as evidenced by the singlet in the ^{31}P nmr spectrum.

Preparation of $\text{PEt}_3\text{AuL}^+\text{ClO}_4^-$

All compounds 19–25 were prepared using routes analogous to literature methods [16]. A sample preparation is given.

50 mg (0.143 mmol) of PEt_3AuCl was dissolved in methanol (10 ml). To this solution 29.4 mg of AgClO_4 is added. After stirring for 5 min the flocculent precipitate of AgCl is removed by filtration through celite. 13.3 mg (0.143 mmol) of $\text{C}_6\text{H}_5\text{NH}_2$ was added to the filtrate. The solvent was removed and replaced with CDCl_3 . ^{31}P nmr spectral data showed a clean singlet indicative of pure $\text{PEt}_3\text{Au}(\text{H}_2\text{NC}_6\text{H}_5)^+\text{ClO}_4^-$.

Preparation of Albumin, HSA and BSA

0.100 g of the protein was dissolved in 1.5 ml of deionized, distilled water. 70 μl of 14.25 *M* $\text{HSCH}_2\text{-CH}_2\text{OH}$ was added and stirred for 1/2 h. The solution was dialyzed against 4 l of deoxygenated 50 *mM* TRIS buffer (pH = 8.5). The protein was lyophilized and stored under N_2 at 0 $^\circ\text{C}$.

Preparation of Sulphydryl Blocked HSA and BSA

The protein was reduced as above. 100 mol excess of iodoacetamide was added. The pH was raised to 8.5 and the solution was stirred for 1 h. The solution was dialyzed against 8 l of cold H_2O , lyophilized and stored under N_2 at 0 $^\circ\text{C}$.

Reactions of HSA, BSA with PEt_3AuCl

50 μl of 67 mg/ml solution of the protein was diluted with 1.0 ml D_2O . To this solution was added 50 μl of 0.48 *M* PEt_3AuCl in methanol. ^{31}P nmr data were recorded on the resulting solutions.

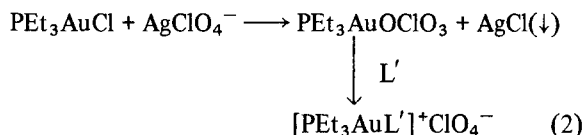
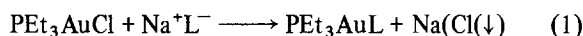
Samples for fluorescence studies were prepared as above followed by 1 h of incubation at 25 $^\circ\text{C}$ and centrifugation. Fluorescence spectra were recorded on the supernatants. Precise protein concentration in the supernatants was determined by the Bio-Rad Coomassie Blue Microprotein assay.

Results and Discussion

Model Compounds

The linear gold(I) compounds prepared were of formulae PEt_3AuL and $[\text{PEt}_3\text{AuL}']^+\text{ClO}_4^-$, where L

is either thiolate, phenolate or carboxylate and L' is thioether, amine, or imidazoles. The former neutral compounds were prepared by simple nucleophilic displacement of chloride from PEt_3AuCl [13–15] (eqn. 1). The latter salts were prepared by removal of chloride by AgClO_4 followed by addition of a neutral group V or group VI donor molecule [16] (eqn. 2). These previously known routes were used to prepare all of the compounds in Table I:



All of the gold compounds were characterized by ^1H decoupled ^{31}P nmr spectra in chloroform. The purity of the complexes was indicated by the presence of only clean singlets in the ^{31}P nmr spectra. Observed chemical shifts relative to 85% H_3PO_4 are given in Table I.

The ligands employed in the preparation of these compounds were selected so as to mimic conceivable ligation sites of the ' PEt_3Au ' moiety to biomolecules. Thus only the various forms of N, S and O donor molecules have been utilized.

The ^{31}P chemical shifts of all the gold compounds are approximately 50 ppm downfield of the free phosphine. This complexation shift results from the removal of electron density from the P atom upon coordination to the metal. Such effects have been described for other metal–phosphine complexes [17, 18]. The ^{31}P chemical shifts of the compounds reported herein are dependent on the nature of the ligand trans to the PEt_3 [19]. The following order is observed $^-\text{SR} > \text{S}^{2-} > \text{Cl}^- > \text{NR}_3 \sim \text{imidazole} > ^-\text{OR} \sim ^-\text{O}_2\text{CR} > \text{SR}_2$.

This ordering reflects the trans influence of the ligands.

Thiolates and sulfide are stronger σ donors than amines, alkoxides or carboxylates. They are also better π acceptors, facilitating electron density removal from the P atom and thus the observed downfield shift. Presumably the weak σ donor ability of thioethers prohibit significant metal–ligand π overlap. Thus the chemical shift of the P atom in these complexes appears at high field.

The variation of the ^{31}P chemical shift as a function of solvent is reported for PEt_3AuCl (Table II). Generally a monotonic relationship is observed between solvent dipole moment and ^{31}P chemical shift. However, the relationship is certainly not linear. The range of variation for the solvents reported herein is 2 ppm. It appears that solvation by polar solvents provides an interaction with the gold complex that results in a deshielding of the P atom. Thus to some

extent the chemical shift reflects the polarity of the environment of the 'PEt₃Au' moiety.

Protein Studies

Albumin (HSA or BSA) is treated with mercaptoethanol for a short period of time [20]. This ensures reduction of the one exterior sulfhydryl residue leaving the internal disulfides intact. The reaction of PEt₃AuCl with the albumin was monitored by ³¹P nmr. The spectra were obtained after treatment of an aqueous solution of the protein with a methanolic solution of PEt₃AuCl. The spectra showed a sharp signal at 34.2 and 34.9 ppm for HSA and BSA respectively (Table III, Fig. 1). A weaker broader peak was also observed at about 33 ppm. The samples were centrifuged to remove precipitated gold complex. The supernatants gave identical spectra to those described above. The chemical shift of the singlets is indicative of Au-S binding, based on comparison to the model compounds. This result is supported by preliminary Mössbauer data mentioned briefly by Brown and Smith [3]. The absence of additional intense resonances implies a considerable specificity of gold for binding to this exterior S residue.

The model compounds exhibited a relationship between solvent polarity and chemical shift. Based on extrapolations to H₂O, the chemical shift observed for the 'PEt₃Au' bound to protein was upfield of expected values. We suggest that this upfield shift reflects an environment of lower polarity than the bulk solvent, that is the SH group to which the gold

TABLE II. Solvent Dependence of ³¹P Nmr Data for PEt₃-AuCl.

Solvent	δ (ppm)
CDCl ₃	31.7
(CD ₃) ₂ CO	32.7
CD ₃ OH	33.1
CD ₃ CN	33.2
(CD ₃) ₂ SO	33.7

TABLE III. Protein-PEt₃AuCl Interactions: ³¹P Nmr Data^a.

Protein	δ (ppm)
Albumin (HSA)	34.2(s) 33(weak, broad)
Albumin (BSA)	34.9(s) 33(weak, broad)
SH-blocked HSA	33(broad) 31(broad)
SH-blocked BSA	33(broad) 31(broad)

^aSpectra recorded in D₂O at T = 25 °C.

binds is located in a hydrophobic region of the protein.

The SH site of albumin can be blocked by reaction with iodoacetamide [20]. Reaction of PEt₃-AuCl with the blocked proteins was performed in an analogous manner to that described for albumin. Two broad resonances at 33 and 31 ppm upfield of the reference were observed in the ³¹P nmr spec-

TABLE I. ³¹P Nmr Data for Gold Compounds.

L	δ (ppm) ^a	L	δ (ppm) ^a
(i) Complexes of the form PEt ₃ AuL			
1 Cl ⁻	31.7	10 EtO ₂ CCH(NH ₂)CH ₂ S ⁻	35.4 ^b
2 PhO ⁻	27.2	11 HO(CH ₂) ₂ S ⁻	37.9
3 CH ₃ CO ₂ ⁻	26.9	12 Na ⁺ O ₂ CCH ₂ S ⁻	38.2
4 CH ₃ O ₂ C(NHCOCH ₃)CHCH ₂ C ₆ H ₄ O ⁻	27.4 ^b	13 EtO ₂ CCH ₂ S ⁻	37.8
5 H ₂ NCH ₂ CO ₂ ⁻	27.6	14 NBu ₄ ⁺ O ₂ CCH(NH ₂)C(CH ₃) ₂ S ⁻	37.8 ^b
6 PhS ⁻	37.1	15 C ₁₄ H ₁₉ OS ⁻	36.0 ^b
7 BuS ⁻	37.6	16 C ₆ H ₁₁ O ₅ S ⁻	37.5 ^b
8 ⁻ S(CH ₂) ₂ S ⁻	36.1	17 NBu ₄ ⁺ O ₂ CCH(CH ₂ CO ₂ ⁻ NBu ₄ ⁺)S ⁻	36.2 ^b
9 o-H ₂ NC ₆ H ₄ S ⁻	37.3	18 (CH ₃) ₂ NCS ₂ ⁻	34.1
(ii) Complexes of the form PEt ₃ AuL ⁺ ClO ₄ ⁻			
19 EtNH ₂	30.6	22 CH ₃ O ₂ CCH(NH ₂)CH ₂ C ₃ H ₃ N ₂	29.9 ^b
20 PhNH ₂	31.2	23 CH ₃ O ₂ CCH(NH ₂)CH ₂ CH ₂ SCH ₃	24.3 ^b
21 C ₃ H ₄ N ₂	30.0 ^b	24 Bu ₂ S	24.7
		25 none	27.7 ^b
(iii) Others			
26 PEt ₃ AuCl ₃	54.5	27 (PEt ₃ Au) ₂ S	33.0

^aSpectra were recorded in CDCl₃ at 25 °C. ^bLigands were prepared as follows: 4, N-acetyl tyrosine ethyl ester plus NaOCH₃; 10, cysteine ethylester plus NaOCH₃; 14, penicillamine plus 2 NBu₄OH; 15, thioglucose tetraacetate plus NaOCH₃; 16 thioglucose plus NaOCH₃; 17, thiosuccinic acid plus 3 NBu₄OH; 21, imidazole; 22, histidine methyl ester; 23, methionine methyl ester; 25, no ligand was added.

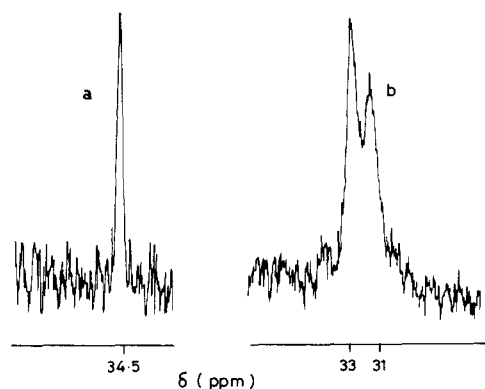


Fig. 1. ^1H decoupled ^{31}P nmr spectra of (a) albumin (HSA) after reaction with PET_3AuCl , (b) blocked albumin (HSA), after reaction with PET_3AuCl .

tra of the reaction mixtures. These signals indicate clearly the presence of non-specific binding to the protein by PET_3AuCl . This was also observed to a much lesser extent in the spectra of gold-albumin mixtures. These ^{31}P nmr experiments clearly demonstrate the affinity and specificity of the gold-phosphine complex for the sulfhydryl group in albumin.

Fluorescence properties have been studied to examine biomolecule interactions in a large number of protein systems [21, 22]. We recorded fluorescence spectra of albumin alone and following reaction with PET_3AuCl to study the effect of gold binding to the SH residue. Spectra were recorded using an excitation wavelength of 280 nm. No shift in the emission maximum was observed on addition of PET_3AuCl to the albumin, however, a 12% decrease in signal intensity was seen (Fig. 2). Such a change in intensity could be interpreted as heavy metal quenching of the fluorescence process [21, 22]. However, this

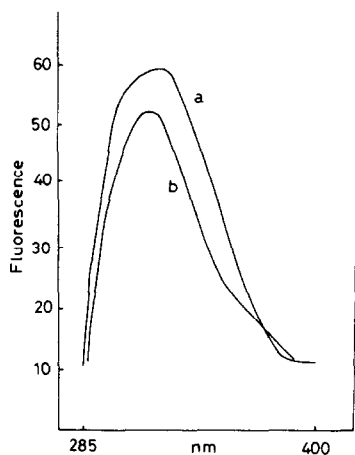


Fig. 2. Fluorescence spectra of (a) albumin, (b) albumin after reaction with PET_3AuCl . Protein concentrations in both cases are 2.92 mg/ml.

seems unlikely as the fluorescence of albumin arises from the tryptophan residue which is a considerable distance from cysteine 34, the gold binding site [23]. An alternative interpretation is that gold binding causes a protein conformational change that results in the observed decrease in fluorescence. The degree and nature of such changes are unknown.

In light of our results it is tempting to speculate that binding of gold to a sulfhydryl residue and an associated protein conformational change are two events leading to drug action. The specific protein system or subsequent steps in such a mechanism are yet to be determined.

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References

- 1 A. Lorber and T. M. Simon, *Gold Bull.*, 12, 149 (1979).
- 2 P. J. Sadler, *Structure and Bonding*, 29, 171 (1976).
- 3 D. H. Brown and W. E. Smith, *Chem. Soc. Rev.*, 9, 217 (1980).
- 4 B. Sutton, 'Platinum, Gold and Other Metal Chemotherapeutic Agents, Chemistry and Biochemistry', *Amer. Chem. Soc. Symp. Series* 209, Ed. S. J. Lippard, 355 (1983).
- 5 M. T. Razi, G. Otiko and P. J. Sadler, *ibid.*, 371 (1983).
- 6 R. C. Elder, M. K. Eidsness, M. J. Herg, K. G. Tepperman, C. F. Shaw III and N. Schaeffer, *ibid.*, 385 (1983).
- 7 D. H. Brown and W. E. Smith, *ibid.*, 401 (1983).
- 8 B. M. Sutton, E. McGusty, D. T. Walz and M. J. DiMartino, *J. Med. Chem.*, 15, 1095 (1972).
- 9 J. Weinstock, B. M. Sutton, G. Y. Kuo, D. J. Walz and M. J. DiMartino, *J. Med. Chem.*, 17, 139 (1974).
- 10 A description of the classic bioinorganic approach to modelling metalloenzymes and metal ions in biology is given by J. A. Ibers and R. H. Holm, *Science*, 209, 223 (1980).
- 11 F. G. Mann, A. F. Wells and D. Purdie, *J. Chem. Soc.*, 1928 (1938).
- 12 R. J. Puddephatt and P. J. Thompson, *J. Chem. Soc. (D)*, 1810 (1975).
- 13 C. Kowala and J. M. Swan, *Aust. J. Chem.*, 19, 547 (1966).
- 14 C. Kowala and J. M. Swan, *Aust. J. Chem.*, 19, 555 (1966).
- 15 G. E. Coates, C. Kowala and J. M. Swan, *Aust. J. Chem.*, 19, 539 (1966).
- 16 R. J. Puddephatt, 'Chemistry of Gold', Elsevier, New York, 1978.
- 17 N. C. Payne and D. W. Stephan, *J. Organomet. Chem.*, 221, 203 (1981).
- 18 J. F. Nixon and A. Pidcock, *Ann. Rev. NMR Spectrosc.*, 5B (1973).
- 19 P. G. Jones and A. F. Williams, *J. Chem. Soc. (D)*, 1430

- (1977).
- 20 B. Mutus and J. Amponsah, *Biochem. Biophys. Res. Commun.*, in press.
- 21 S. Unenfriend, 'Fluorescence Assay in Biology and Medicine', Vol. 1, Academic Press, New York, 1962.
- 22 S. Unenfriend, *ibid.*, Vol. 2, Academic Press, New York, 1969.
- 23 J. R. Brown, 'Albumin Structure, Function and Uses', Ed. V. M. Rosenoer, M. Oratz, M. A. Rothschild, Pergamon Press, Toronto, 1977.