Pergamon

0731-7085(94)00118-9

Journal of Pharmaceutical & Biomedical Analysis, Vol. 13, No. 2, pp. 99–102, 1995
Copyright © 1995 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0731-7085/95 $9.50 + 0.00

A comparative study of the interaction of Myocrisin with albumin and γ-globulin

C. McDONALD, P. MORTIMER, G. McALLISTER, P.E. GARDINER,† J. REGLINSKI* and W.E. SMITH

Department of Pure and Applied Chemistry, Strathclyde University, 295 Cathedral St, Glasgow G1 1XL, UK

Abstract: The reactions of Myocrisin with albumin and γ-globulin have been investigated using atomic absorption spectrometry and gel filtration. Albumin rapidly binds gold up to the levels predicted from the concentration of free sulphydryl groups present in the protein. However, in the presence of glutathione, gold incorporation is increased, suggesting that *in vivo*, free thiols (glutathione, thiomalate) may facilitate gold uptake by the protein. In comparison, γ-globulin is found to be capable of binding up to one atom of gold per molecule of protein in a slow reaction which requires high Myocrisin concentrations.

Keywords: *Gold; Myocrisin; albumin; γ-globulin.*

Introduction

Gold therapy remains one of the few effective treatments for rheumatoid arthritis. Active compounds have been developed which allow it to be administered orally (Auranofin) and intravenously (Myocrisin) [1]. Irrespective of which route is preferred clinically, it is clear that the bulk of the gold is transferred quickly to ligand sites on the circulating cells [2] and on plasma proteins [3]. The latter, principally albumin, act as a major long term pool for a large proportion of the administered gold present in the blood [3]. The metal protein complex formed has been described as a pro-drug [4], which facilitates the transport of gold to the various regions of the body including the synovial cavity, where it is believed to be active. It is, therefore, important that the number of binding sites available on each of the plasma proteins is documented and their reactivity understood.

Thus far, studies of the interaction of gold with the plasma proteins has focused mainly on the chemistry of albumin and in particular on cysteine-34 [5, 6] located in the first domain [7]. It remains unclear if there is a second, weaker metal binding site for gold on albumin. For example, an interaction of gold with the copper or nickel binding sites [8, 9], would allow albumin to transport a second gold unit. Clinical studies on the determination of gold in plasma using electrophoresis have identified the presence of some Myocrisin loosely bound to albumin [1], separate from that bound to cysteine-34. This gold was postulated to reside in one of the pockets in the protein which are commonly occupied by other drugs. Although the amount of this "second type" of gold varied from patient to patient, it was usually of the order of 15% of the total [1]. The presence of a second weak binding site for the metal would give rise to a more active pool of gold which will be transferred more easily and would subsequently have more clinical relevance.

Of the other plasma proteins, it is thought that gold will bind to the selenium site on glutathione peroxidase [10]. Clinical studies indicate the presence of gold bound to γ-globulin, although at lower concentrations than in albumin. As the globulins lack a reactive sulphydryl function, the most likely reaction is the exchange between the gold–thiolate bond and disulphide bridges [11, 12] in the protein, possibly in the swing region.

In this study we have quantitatively investigated the ability of gold to bind to both albumin and γ-globulin to try and establish

* Author to whom correspondence should be addressed.
† Present address: Division of Chemistry, Sheffield Hallam University, Sheffield.

how many binding sites exist for gold on the circulating plasma proteins and whether there are any priorities to their occupancy.

Experimental

Albumin and γ-globulin (Cohn fraction 10) were purchased from the Sigma Chemical Co. (Poole, UK). Myocrisin was purchased from the Aldrich Chemical Co. (Poole, UK).

Estimation of the sulphydryl content of albumin and γ-globulin

The sulphydryl content of γ-globulin and two separate batches of albumin were assayed using 5,5′-dithiobis-(2-nitrobenzoic acid), Ellmans reagent, in the standard manner [13]. Although γ-globulin is known to have no free sulphydryl functions, the protein was assayed to ensure that there were no major sulphydryl-containing impurities in the purchased material.

As albumin exists as a heterogeneous mixture in three forms (free sulphydryl, mixed disulphide with cysteine, and mixed disulphide with glutathione), different batches of albumin obtained commercially have differing amounts of free thiol. Each batch requires to be assayed [13] before use. The amount of free thiol may be expected to range between 0.3 -SH groups per protein and 0.7 -SH groups per protein.

Preparation and separation of gold-modified proteins

Myocrisin and albumin were mixed in equimolar amounts in a small volume (2 ml, 5×10^{-4} M) of phosphate buffered saline, pH buffered to 7.4 (PBS) and allowed to react for 1 h. The solution was applied to a Sephadex G25 column (2.6 × 40 cm) and eluted with PBS. One millilitre fractions were collected and analysed for protein by UV-spectrophotometry, and for gold by graphite furnace atomic absorption spectrometry as described elsewhere [14], using a Phillips PU 9100 spectrophotometer.

The preparation of the gold modified γ-globulin follows that described for albumin, except that higher concentrations of gold were also used (1:1, 1:2, 1:3, 1:5, 1:7 mole:mole, protein:gold). Due to the slower reaction of γ-globulin, the mixtures were allowed to react for 7 days prior to separation and analysis. Due to the prolonged incubation period in this study, the samples were submitted to sterile filtration into sterile flasks in a laminar flow cabinet. The flasks were firmly stoppered to prevent interference from bacteria.

Results and Discussion

The activity of gold in rheumatoid arthritis must be governed to some extent by the chemistry of the species present in solution. Thus, although Auranofin and Myocrisin both produce beneficial effects clinically, the changes which occur with Auranofin in the digestive tract prior to absorption, and transfer to the macromolecules within the circulation, makes it difficult to clearly identify the important active species. Myocrisin, however, is administered intramuscularly, some of it appearing in the plasma in an unchanged form [1]. This makes the *in vitro* reaction of Myocrisin with plasma proteins a key structural point of reference. The structure of Myocrisin is known to be polymeric involving bridging thiolates [11, 15, 16]. It is susceptible to rapid ligand exchange and, in the presence of a donor, to degradation [15]. This chemistry undoubtedly facilitates the transfer of polymeric gold thiolate ([AuSR], RS = thiomalate) moieties to the cysteinyl residue on albumin and possibly other soft binding sites on the plasma proteins.

It is easy to produce an *in vitro* simulation for the early events involved in the action of Myocrisin by mixing the drug with plasma proteins. The proteins can be saturated with gold by using excess Myocrisin and the modified protein generated separated by gel-filtration. The amount of gold incorporated into the protein fraction can be assayed by atomic absorption spectrometry and correlated, where applicable (i.e. albumin), with the corresponding thiolate level for the unmodified protein determined using the standard Ellmans assay [13].

Albumin studies

It can be seen from Fig. 1 that there is a good separation of the modified albumin from the excess gold, and that gold is incorporated into the modified protein at levels (0.31 ± 0.05 gold atoms per molecule of protein) consistent with the amount of free thiol (0.32 ± 0.02 -SH groups per protein). Although the assay suggests that there is little gold incorporation above the level predicted on the basis of the number of sulphydryl binding sites available,

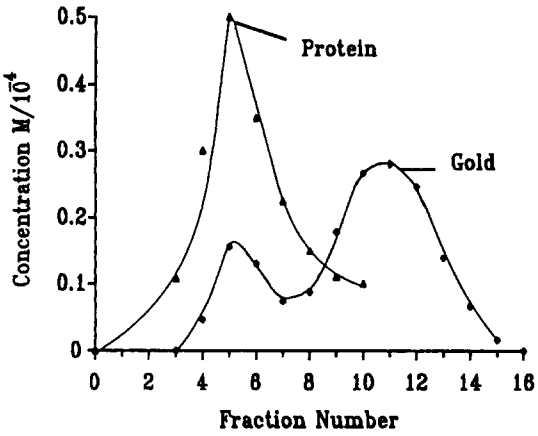

Figure 1
The concentration of albumin and gold in the 1-ml fractions obtained from the gel filtration column. Protein concentrations were determined by UV-spectrophotometry (280 nm) and the gold concentration by graphite furnace atomic absorption spectrometry.

there could still be a small amount of gold adsorbed at other sites.

Using a different batch of albumin (0.58 ± 0.03 -SH groups per protein), samples of gold modified protein were prepared in the presence of excess glutathione. Although this would degrade the polymeric gold complex [15], it would not prevent gold incorporation at cysteine-34. However, it would be expected to prevent gold incorporation at the weaker binding sites by providing a more apposite ligand (glutathione) for gold in solution. Analysis of these samples did not show any diminution of gold incorporation but curiously indicated that gold incorporation increased from 0.65 ± 0.05 to 0.82 ± 0.05 gold atoms per molecule of protein. This suggests that in the presence of a reducing agent, glutathione, it is possible to generate extra sites on the protein by cleaving the mixed disulphides with cysteine and glutathione in the heterogeneous albumin mixture.

γ-Globulin studies

Although γ-globulin does not contain a free sulphydryl function and, therefore, cannot bind gold via rapid ligand exchange with a free thiolate, the data (Fig. 2) indicates that the protein is still capable of sequestering gold from Myocrisin. For this reaction to occur, however, prolonged incubation times (7 days) and relatively high gold concentrations are required. This behaviour is indicative of an interaction of Myocrisin with γ-globulin disulphide bridges, a reaction known to be slow for simple disulphides [11]. The observed

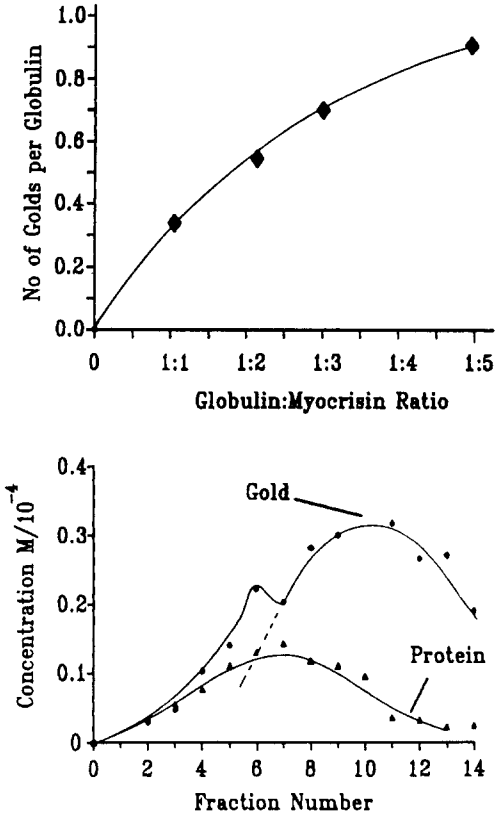

Figure 2
Above: gold incorporation into γ-globulin as a function of the molar ratio of γ-globulin: Myocrisin in the reaction mixture. Below: the concentration of γ-globulin and gold in the 1-ml fractions obtained from the gel filtration column. Protein concentrations were determined by UV-spectrophotometry (280 nm) and the gold concentration by graphite furnace atomic absorption spectrometry.

protein–gold interaction would seem to be stoichiometeric, each γ-globulin being capable of binding up to one gold atom per molecule of protein.

Concluding Remarks

It would seem unlikely that there is a second weak binding site for gold on albumin. However, the data suggests that under reducing conditions it is possible to achieve incorporation of gold into the protein at levels higher than initially expected. This may go some way to explain why certain workers have implied that a second site exists. Other studies have highlighted the importance of the thiolate ligand (thiomalate, thioglucose) used to prepare active complexes [1]. This study suggests that the incorporation of gold into protein is in some way facilitated by the removal of free ligand by albumin mixed disulphides.

Compared to albumin, the interaction of gold with γ-globulin is slow and requires a high gold concentration. However, distribution studies in a number of patients show that the ratio of albumin gold to the gold found on the globulins does not change appreciably as the plasma gold levels rise [12]. At maximum therapeutic dose, the gold level is well below the saturation level of either the rapidly reacting albumin site or the slowly reacting γ-globulin site, with the former accounting for roughly 80% of the total gold observed. Thus the amount of γ-globulin affected will increase as the effective plasma gold concentration increases, possibly causing a greater immunological effect around 12 weeks. If γ-globulin modification reflects a modification of related immunologically useful proteins such as rheumatoid factor, and there is nothing protein specific in the disulphide exchange proposed, then a response time about the same as that required to build up the gold concentration would give suitable kinetics.

References

[1] W.E. Smith and J. Reglinski, *Persp. Bioinorg. Chem.* **1**, 183–208 (1991).
[2] J.M. Campbell, J. Reglinski, W.E. Smith, D. Porter and R.D. Sturrock, *Ann. Rheum. Dis.* **51**, 969–971 (1992).
[3] H. Kamel, D.H. Brown, J.M. Ottaway and W.E. Smith, *Analyst* **12**, 645–657 (1977).
[4] C.F. Shaw, III, B. Schliesman, J. Xiao, J. Locke, M. Starich and D. Fetter, *J. Inorg. Biochem.* **43**, 594 (1991).
[5] A.A. Isab, A.L. Hormann, D.T. Hill, D.E. Griswold, M.J. DiMartino and C.F. Shaw, III, *Inorg. Chem.* **28**, 1321–1326 (1989).
[6] C.F. Shaw, N.A. Schaeffer, R.C. Elder, M.K. Eidness, J.M. Trooster and G.H.M. Calis, *J. Am. Chem. Soc.* **106**, 3511–3521 (1984).
[7] X.M. He and D.C. Carter, *Nature* **58**, 209–214 (1992).
[8] J. Dolovich, S.L. Evans and E. Nieboer, *Br. J. Indust. Med.* **41**, 51–55 (1984).
[9] J. Masuoka, J. Hegemaur, B.R. Vandyke and P. Saltman, *J. Biol. Chem.* **268**, 21533–21537 (1993).
[10] J. Chaudiere and Al. L. Tappel, *J. Inorg. Biochem.* **20**, 313–325 (1980).
[11] J. Reglinski, S. Hoey and W.E. Smith, *Inorg. Chim. Acta* **152**, 261–264 (1988).
[12] H. Capell, D.H. Brown and W.E. Smith, *Inorg. Chim. Acta* **106**, L23–L24 (1985).
[13] G.L. Ellman, *Arch. Biochem. Biophys.* **82**, 70–77 (1959).
[14] J. Egila, D.L. Littlejohn, W.E. Smith and R.D. Sturrock, *J. Pharm. Biomed. Anal.* **10**, 639–644 (1992).
[15] A.A. Isab and P.J. Sadler, *J. Chem. Soc. Dalton Trans.* 135–141 (1982).
[16] R.C. Elder, K. Ludwig, J.N. Cooper and M.K. Eidness, *J. Am. Chem. Soc.* **107**, 5024–5025 (1985).

[*Received for review 2 February 1994; revised manuscript received 8 August 1994*]