The Reactions of Hemopexin and Liver Fatty Acid-binding Protein (L-FABP) with Aurothiomalate

C. FRANK SHAW III*, FRANCES BEREZIN

Department of Chemistry, University of Wisconsin-Milwaukee, Milwaukee, Wis. 53201, U.S.A.

STYLIANI H. VINCENT[a,b] and URSULA MULLER-EBERHARD[a,b,c]

Departments of Pediatrics[a], Pharmacology[b] and Biochemistry[c], The New York Hospital-Cornell University Medical College, New York, N.Y. 10021, U.S.A.

(Received February 19, 1988)

Abstract

We investigated whether gold(I) binds to hemopexin, Hx, a serum heme carrier with a molecular weight similar to that of serum albumin, or to L-FABP, a liver cytosolic heme- and fatty acid-binding protein whose molecular weight is similar to that of metallothionein. These proteins and, for comparison, similar concentrations of bovine albumin were incubated with sodium aurothiomalate and then fractionated by gel exclusion chromatography. At 16 μM of Hx and gold the ratio of gold bound to Hx was 0.30 ± 0.04. The corresponding ratio for albumin was 0.98 ± 0.04 per mercaptalbumin. Considering the much lower serum levels of Hx compared to albumin it is unlikely that Hx plays a role in serum gold binding and transport. L-FABP also binds gold: at 52 μM L-FABP and 108 μM gold, the gold to protein ratio was 0.55 ± 0.05. The corresponding ratio for albumin under identical conditions was 1.18 ± 0.08 per mercaptalbumin. L-FABP failed to bind zinc or cadmium, two other metals bound by metallothionein.

Introduction

The mechanisms by which essential or xenobiotic metals are recognized, transported, sequestered or incorporated into biomolecules are known with certainty in only a few well studied cases [1]. In many cases, xenobiotic metal species may bind to and be transported by biochemical macromolecules normally associated with other processes or functions. For example, the medicinally used metal ion gold(I) binds to metallothionein [2], a protein with functions in zinc and copper metabolism [3]. In the serum, many metals, essential and xenobiotic, bind to serum albumin [4]. Because metallothionein and serum albumin have well established roles in the biochemistry of many metals, there is often an implicit but not necessarily valid assumption that any metal in the 60 000 M_r band during chromatography of serum or the 12 000 M_r band of liver or kidney cytosol is bound to albumin or metallothionein, respectively.

The present investigation was undertaken to explore whether hemopexin, Hx[§] [5], a 60 kDa protein present in serum at ≃1/60 the concentration of albumin, might compete with albumin for gold and whether the 14 kDa liver fatty acid-binding protein, L-FABP [6–8], might bind gold, zinc or cadmium. Hx is the specific heme carrier in serum with an affinity for heme ≃10^{13} M^{-1} [9]. It also binds, but with much lower affinity, porphyrins [5], bilirubin [10], cobinamide and vanadate [11]. L-FABP (which has also been referred to as heme-binding protein, HBP [7, 8] and Z protein [12]) binds several organic anions, including heme for which it has the highest affinity. It is an abundant protein comprising as much as 5% of liver cytosol protein. Bovine serum albumin (used instead of human serum albumin because BSA has a more reproducible content of mercaptalbumin, the gold binding component) was examined for comparison. The clinically used antiarthritic drug gold sodium thiomalate (abbreviated $Au(STm)_{1+x}$ because it contains a slight excess (5–15%) of thiomalate over gold) was used for these studies.

Experimental

Reagents

Sodium aurothiomalate ($(Au(STm)_{1+x}$) and 4,4-dithiopyridine (PDS) were purchased from Aldrich Chemical Co.; 5,5-dithiobis(2-nitrobenzoic acid)

*Author to whom correspondence should be addressed.

[§] Abbreviations: $Au(STm)_{1+x}$, sodium aurothiomalate; BSA, bovine serum albumin; DTNB, 5.5-dithiobis(2-nitrobenzoic acid); Hx, hemopexin; L-FABP, liver fatty acid-binding protein; PDS, 4,4'-dithiopyridine.

0020-1693/88/$3.50

(DTNB) and Sephadexes G-25 and G-50, from Sigma Chemical Co. Reagent grade or better inorganic chemicals were purchased from standard suppliers.

Proteins

Hx was purified from human plasma by heme agarose affinity chromatography [13], modified as previously described [14], and L-FABP was purified from liver cytosol of Sprague-Dawley rats by sequential chromatography [7]. Hx concentration was measured using its molar extinction coefficient, (ϵ_{280} = 125 000 l/mol/cm) [5]; L-FABP concentration was determined by the Bradford method [15]. Bovine serum albumin (BSA) was purchased from Boehringer Mannheim Biochemicals. Aliquots of BSA and L-FABP stock solutions were diluted appropriately and analyzed for reactive sulfhydryl group using published procedures employing DTNB [16] and PDS [17].

Metal Binding Studies

Stock solutions of Hx and $Au(STm)_{1+x}$ were added to sufficient buffer (50 mM Tris-HCl/150 mM KCl/3mM NaN_3) to make 1.0 ml solutions containing 16 μM Hx and Au. Aliquots were fractionated over Sephadex G-25. The fractions were analyzed for gold by atomic absorption spectroscopy and for protein by its absorbance at 280 nm. For comparison, the same concentration of BSA was reacted under identical conditions.

A stock solution of L-FABP (0.5 mg/ml) in 100 mM NH_4HCO_3 buffer, pH 7.6, was used to prepare solutions containing 52 μM L-FABP and 108 μM AuSTm. Aliquots were fractionated over Sephadex G-50 and the fractions were analyzed for gold and protein content. The reaction was compared to that of serum albumin (52 μM) and gold (108 μM) under identical conditions. Similar binding studies were conducted using 27 μM L-FABP and 54 μM $Cd(OAc)_2$ or $Zn(OAc)_2$.

Results

Hx reacts with equimolar $Au(STm)_{1+x}$ to form a gold protein adduct, which withstands chromatography over Sephadex G-25 (Fig. 1). Hx elutes at the void volume as a single well-defined peak. Gold elutes as two overlapping peaks. The smaller is a shoulder on the main peak and is coincident with the Hx peak. The larger, trailing peak elutes after the Hx band, indicating that the majority of the gold is not protein-bound. The profile of the eluted gold clearly establishes that it is the superposition of two independent gold species. The association of the smaller component with Hx indicates that incomplete reaction takes place between

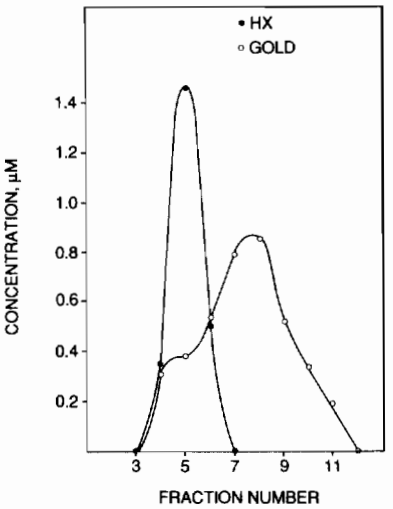

Fig. 1. Chromatography of an equimolar mixture of Hx and $AuSTm_{1+x}$ after incubation for 19 h. Sephadex G-25 (1 × 15 cm column) eluted with 100 mM NH_4HCO_3 buffer, pH 7.6.

$Au(STm)_{1+x}$ and the protein. The ratio of gold to hemopexin for two samples chromatographed at successive times was 0.30 ± 0.04. A slight increase in the gold to Hx ratio after 19 hours was not considered to be significant.

For comparison, the reaction of gold with BSA, which is known to bind $Au(STm)_{1+x}$ very effectively, was conducted under similar conditions of concentrations, buffer and reaction time. Under the conditions used here, $AuSTm_{1+x}$ reacts only with the mercaptalbumin (AlbSH) component of BSA [18], which is a complicated, microheterogeneous mixture *in vivo*. The mercaptalbumin content of the BSA sample, 0.50, was determined immediately before the gold reaction was conducted [16]. The stoichiometry of the gold–albumin complex, 0.49 ± 0.02, corresponds to a stoichiometric reaction, 0.98 ± 0.04, at the cys-34 sulfhydryl group of AlbSH [18].

L-FABP was reacted with a 2:1 molar ratio of $AuSTm_{1+x}$. Aliquots were fractionated over Sephadex G-50. The gold bound to L-FABP was more completely resolved from the unbound, low-molecular weight gold (Fig. 2). In the absence of a well-established molar extinction coefficient, total recovery of the protein was assumed in order to calculate the gold–protein ratios in Table I. Using this approach, the gold–protein ratio was found to be 0.55 ± 0.05. The ability of the L-FABP to bind gold(I) is consistent with the presence of a cysteine residue at position 69 [19]. The thiol reagents DTNB and PDS reacted very slowly with L-FABP, even at room temperature. DTNB reacted completely (0.98 ± 0.01), while PDS reacted incompletely (0.77 ± 0.01). The same structural factors

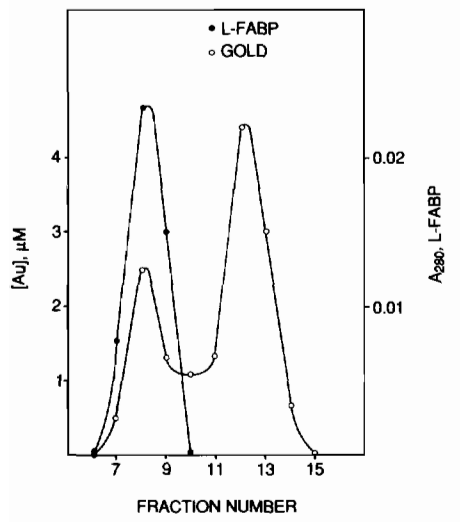

Fig. 2. Chromatography of an 2:1 molar ratio of $AuSTm_{1+x}$ and L-FABP fractionated over Sephadex G-50 (1 × 19 cm column) and eluted with 100 mM NH_4HCO_3 buffer, pH 7.6.

TABLE I. Binding Ratios of Gold to HX or L-FABP

Protein (μM)	$Au(STm)_{1+x}$ (μM)	Au/ Protein[a]	Au/ Sulfhydryl[a]
HX (16)	16	0.30 ± 0.04	
L-FABP (52)	108	0.55 ± 0.05	0.56 ± 0.05
BSA (16)	16	0.49 ± 0.02	0.98 ± 0.04[b]
BSA (52)	108	0.59 ± 0.04	1.18 ± 0.08[b]

[a]Mean ± range of two determinations. [b]Ratio of Au to AlbSH; see text for explanation.

rendering these reactions slow and in one case incomplete may render the reaction with $Au(STm)_{1+x}$ incomplete. When BSA was reacted with gold at the same concentrations, it was found to bind slightly more than one gold per mercaptalbumin, 1.18 ± 0.08, which is consistent with previous reports of multiple gold binding sites at cysteine-34 [18].

Since L-FABP is a major constituent of liver cytosol, where cadmium and zinc are bound to metallothionein, a protein of similar M_r upon gel-exclusion chromatography, the possibility that L-FABP binds these two metals was also investigated. After fractionating reaction mixtures containing 27 μM L-FABP and 54 μM $Cd(OAc)_2$ or $Zn(OAc)_2$ over Sephadex G-50, the metals were recovered in the low-molecular-weight fractions and L-FABP in the excluded fractions (not shown), indicating that neither metal is bound to the protein.

Discussion

A comparison of the gold binding abilities of Hx and serum albumin suggests that under normal *in vivo* conditions Hx will not be able to compete with albumin for gold presented as $Au(STm)_{1+x}$. The *in vivo* concentrations of albumin and Hx are in approximately 60/1 ratio. The gold binding observed under equimolar conditions (Table I) establishes a minimum ratio of 3.3/1 for their respective binding constants K_{Alb}/K_{HHx}. Since the albumin was saturated under the conditions used, the ratio is undoubtedly much greater. Using the lower limit of 3.3 for binding constant ratio and the value of 60 for the ratio of protein concentrations predicts a lower limit of 200 for the ratio of albumin bound gold to Hx bound gold under *in vivo* conditions. Thus, as an upper limit, less than 0.5% of the gold in serum should be bound to the Hx. If ligands other than thiomalate are bound to gold, the extent of gold binding to Hx might be altered, but it is unlikely that such a change would suffice to make Hx a significant factor in serum gold transport (except, perhaps, in the analbuminemic rat [20]).

Following the administration of sodium aurothiomalate to rats, a substantial portion of the injected gold can be recovered in the low molecular weight ($M_r \sim 12\,000$) cytosolic fraction of liver and kidney [21]. The major metal-binding protein in this fraction is metallothionein, which has 20 metal-bound sulfhydryl groups. Its basal concentration in the liver is about 0.11 mg/g of wet tissue [22], but it can be increased by induction with gold or other metals. Although L-FABP has only one sulfhydryl group and appears to bind gold less avidly than metallothionein (from which zinc is quantitatively displaced by $Au(STm)_{1+x}$ [2]), the L-FABP concentration in the liver (2.9 mg/g of liver [23]) is almost 30 times that of metallothionein. Thus, the possibility that L-FABPs (or the homologous proteins in the kidney and other tissues) may contribute to the 12 000 M_r gold band can not be discounted and should be further investigated.

In conclusion, we have shown L-FABP, an abundant liver cytosolic protein with an M_r similar to that of metallothionein, and Hx, a specific serum heme carrier both bind $Au(STm)_{1+x}$. Since L-FABP is present in large concentration in liver, further studies of its gold-binding capabilities are warranted. Hx binds $Au(STm)_{1+x}$ less avidly than albumin whose serum concentration is 60 times that of Hx. It is, therefore, unlikely that Hx participates is serum gold transport.

References

1 E.-I. Ochiai, 'Bioinorganic Chemistry: An Introduction', Allyn and Bacon, Boston, 1977.
2 J. E. Laib, C. F. Shaw III, D. H. Petering, M. K. Eidsness, R. C. Elder and J. S. Garvey, *Biochemistry, 24*, 1977 (1985).

3 I. Bremner and R. K. Mehra, *Chem. Scr., 21,* 117 (1983).
4 B. Sarker, *Life Chem. Rep., 1,* 165 (1983).
5 G. DiSabato (ed.), 'Methods in Enzymology', Academic Press, Orlando, in press.
6 R. K. Ockner, J. A. Manning and J. P. Kane, *J. Biol. Chem., 257,* 7872 (1982).
7 S. H. Vincent and U. Muller-Eberhard, *J. Biol. Chem., 257,* 14521 (1985).
8 S. H. Vincent, N. M. Bass, J. M. Sider and U. Muller-Eberhard, *Biochem. Arch., 3,* 443 (1987).
9 Z. Hrkal and U. Muller-Eberhard, *Biochemistry, 10,* 1746 (1971).
10 W. T. Morgan, U. Muller-Eberhard and A. A. Lamola, *Biochim. Biophys. Acta, 532,* 57 (1978).
11 U. Muller-Eberhard and H. H. Liem, in Z. C. Allison (ed.), 'Structure and Function of Plasma Proteins', Vol. 1, Plenum Press, London, 1974, p. 35.
12 A. J. Levi, Z. Gatmaitan and I. M. Arias, *J. Clin. Invest., 48,* 2156 (1976).
13 K. Tsutsui and G. C. Mueller, *Anal. Biochem., 121,* 244 (1982).
14 V. Goldfarb, R. B. Trimble, M. DeFalco, H. Liem, S. A. Metcalfe, D. Wellner and U. Muller-Eberhard, *Biochemistry, 25,* 6555 (1986).
15 M. M. Bradford, *Anal. Biochem., 72,* 248 (1976).
16 G. Ellman, *Arch. Biochem. Biophys., 20,* 70 (1959).
17 R. Ampulski, V. Ayers and S. Morell, *Anal. Biochem., 32,* 163 (1969).
18 C. F. Shaw III, N. A. Schaeffer, R. C. Elder, M. K. Eidsness, J. M. Trooster and G. H. M. Calis, *J. Am. Chem. Soc., 106,* 3511 (1984); S. M. Pedersen, *Biochem. Pharmacol., 36,* 2661 (1987).
19 K. Takahashi, S. Odani and T. Ono, *Eur. J. Biochem., 136,* 589 (1982).
20 K. Sugiyama, T. Emori and S. Nagase, *J. Biochem., 92,* 775 (1982).
21 R. P. Sharma and E. G. McQueen, *Biochem. Pharmacol., 29,* 2017 (1980).
22 E. M. Mogilnicka and J. K. Piotrowski, *Biochim. Pharmacol., 28,* 2625 (1979).
23 N. M. Bass, J. A. Manning and R. K. Ockner, *J. Biol. Chem., 260,* 9603 (1985).