Communication

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J. Am. Chem. Soc., **Just Accepted Manuscript** • DOI: 10.1021/jacs.5b09895 • Publication Date (Web): 13 Nov 2015

Structural insights and the surprisingly low mechanical stability of the Au-S bond in the gold-specific protein GolB

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Supporting Information Placeholder

ABSTRACT: The coordination bond between gold and sulfur (Au-S) has been widely studied and utilized in many fields. However, detailed investigations on the basic nature of this bond are still lacking. A gold-specific binding protein, GolB, was recently identified, providing a unique opportunity for the study of the Au-S bond at the molecular level. We probed the mechanical strength of the gold-sulfur bond in GolB using single molecule force spectroscopy. We measured the rupture force of the Au-S bond to be 165 pN, much lower than Au-S bonds measured on different gold surfaces (~1000 pN). We further solved the structures of apo-GolB and Au(I)-GolB complex using X-ray crystallography. These structures showed that the average Au-S bond length in GolB is much longer than the reported average value of Au-S bonds. Our results highlight the dramatic influence of the unique biological environment on the stability and strength of metal coordination bonds in proteins.

Gold-thiol interactions have been widely used in materials science, molecular biology, and medical engineering. Their versatile applications include self-assembled monolayers (SAM) of bio- or organic molecules on gold, bio-sensing, drug delivery, and gold nanoparticle catalysis[1-4]. The covalent bond between gold and sulfur (Au-S) provides a robust and modifiable linkage that is key to the nanostructures between the gold surface and thiol-containing molecules[5]. To probe the detailed nature of gold-thiol interactions, Gaub and coworkers have performed pioneering work to detect the strength of a single Au-S bond, using atomic force microscopy (AFM)-based single-molecule force spectroscopy (SMFS)6. The structural details and the strength of the Au-S bond in gold-thiol interactions are attracting great attention recently[7]. Encouragingly, Zhang and coworkers successfully employed the AFM-based SMFS method to quantify the strength of individual thiol-gold bonds with different gold surface properties and sample preparation conditions[8]

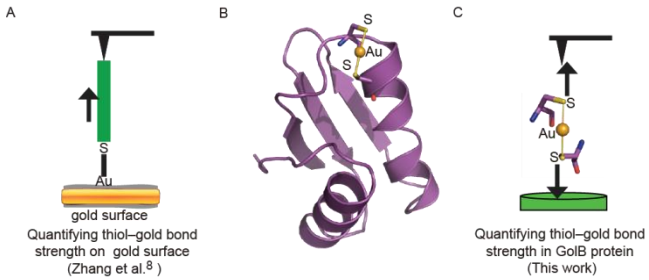

Figure 1. (A) Illustration of the breakage of an isolated Au-S bond on the material's surface (Zhang et al). (B) Crystal structure of Au(I)-bound GolB protein. (C) Illustration of the breakage of an Au-S bond in Au(I)-bound GolB protein (This work).

(Figure 1A). These results suggested that the strength of thiol-gold bonds in self-assembled monolayers depended largely on the chemical environment. However, the chemical properties of the gold surface are difficult to define.

Numerous metalloproteins, including transcription regulators and metallochaperones, are involved in maintaining the delicate homeostasis of cellular concentrations of various metal ions in bacteria[9-14]. Recently, it was discovered that several proteins can bind gold ions and form the Au-S bond selectively[15-18]. Our previous research on wide-type GolB protein from *Salmonella typhimurium* showed that GolB binds Au(I) using a conserved Cys-XX-Cys binding domain and with a much higher affinity than Cu(I)[16]. In gold sensing and resistant bacteria, the *gol* regulon including GolB was regulated by Au(I) and involved with Au -homeostasis *in vivo*[15,19]. In this detoxification process, GolB is in charge of binding excessive free gold ions from cytoplasm and/or periplasm[19]. Thus the Au-S bonds in GolB must be generated and broken in a more dynamic process for releasing gold ions than that in non-protein gold complexes. Exploring the mechanical stability of Au-S bonds in single-

protein settings may provide insights into the structure-function relationship of metalloproteins. However, the structural details of gold coordination and data on the stability of Au-S bonds in GolB are lacking.

Interestingly, Zheng and Li elegantly demonstrated that the mechanical strength of metal-thiolate bonds is extremely sensitive to the chemical and biological environment of the proteins. Using an Fe-binding protein, rubredoxin, as an example, they discovered that both the second-shell binding residues and the pulling directions could significantly affect the strength of Fe-S bonds[20-24]. Because Au-S bonds are generally considered to be covalent bonds and are much stronger than Fe-S coordination bonds, we are intrigued to see how the protein environment could affect the mechanical stability of Au-S bonds.

Here, combining X-ray crystallography and the AFM-based SMFS methods, we studied the structural and mechanical properties of Au-S bonds in a metalloprotein for the first time (Figures 1B and C). We discovered that the Au-S bonds in GolB are unusually longer than those in their inorganic counterparts. They are also longer than the Au-S bond (2.32 and 2.39 Å) found in the Cu metalloregulatory protein, CueR.[13] The mechanical stability of Au-S bonds in GolB was much lower than that of typical covalent bonds. Our results highlight the importance of protein environment on the stability and dynamics of Au-S bonds in single-molecule settings. These findings might provide a basis for understanding the molecular mechanism of gold trafficking mediated by GolB and other metal chaperones.

We used the AFM-based SMFS method to directly rupture single Au-S bonds in GolB and probe their mechanical properties. To unambiguously identify single molecule stretching events, we prepared a chimeric polyprotein $(GB1\text{-}GolB)_4$ (Figure 2A, inset). The well-characterized GB1 was used as a basis to identify the rupture events of GolB. The loop between the two gold-binding cysteine residues in GolB is too short to provide sufficient distance change for AFM to detect after the rupture of Au-S bonds. Therefore, we inserted a fast-folding beta hairpin into the gold binding motif of GolB, following similar protocols introduced by others[22,25,26]. The chimeric polyprotein using the GolB with inserted beta hairpin refers as (beta GB1-GolB)$_4$ hereafter. According to references, because the N- and C- termini of the fast-folding beta hairpin are very close, insertion of this β hairpin does not affect the structure of engineered metalloproteins or their metal center.[22,27] And to evaluate the consequences of the β-hairpin insertion on the structure of gold binding site and the overall of GolB, the Au(I) loading experiment was performed and the results showed the similar absorbance increase in wavelength 250-255nm which was regarded as Au(I) loading to protein(Figure S1A)[17,28]. This indicates that beta hairpin insertion does not impact the Au(I) binding of GolB. Moreover, we also measured the circular dichroism (CD) spectra of $(GB1\text{-}GolB)_4$ and (beta GB1-GolB)$_4$ with and without Au(I) (Figure S1B). The CD spectra are similar for both proteins and Au(I) binding does not change the structure of these proteins. No random coil signature (~ 195 nm) could be detected for (beta GB1-GolB)4, indicating that the protein folds properly. This implies that

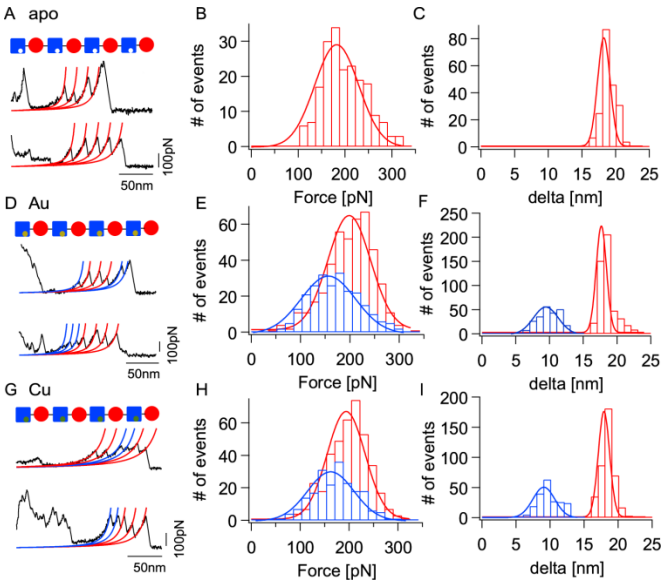

Figure 2. Single molecule force spectroscopy experiments on GolB in the absence and presence of Au or Cu. To probe the mechanical stability of Au-S and Cu-S bonds in GolB (coloured in blue), we engineered a polyprotein (beta GB1-GolB)$_4$, (insets in A, D and G) in which GB1 (coloured in red) is used to provide a "mechanical fingerprint" for single molecule events. A beta hairpin of ~9 nm was inserted between the –CXXC- metal binding motif of GolB to provide sufficient contour length increments for recognition of the rupture of Au-S or Cu-S bonds in the force-extension traces. The representative force-extension traces, unfolding force histogram and distribution of contour length increments for the stretching of (beta GB1-GolB)$_4$ in the apo form are shown in A, B and C, respectively. No unfolding events of apo-GolB were observed, owing to its low mechanical stability. The representative force-extension traces, unfolding force histogram and distribution of contour length increments for the stretching of (beta GB1-GolB)$_4$ charged with Au are shown in D, E and F, respectively. The peaks with contour length increments of ~9 nm are assigned as the rupture events of Au-S bonds. The representative force-extension traces, unfolding force histogram and distribution of contour length increments for the stretching of (beta GB1-GolB)$_4$ charged with Cu are shown in G, H and I, respectively.

the overall secondary structure of GolB did not change with the insertion of the beta hairpin. In single molecule AFM experiments, (beta GB1-GolB)$_4$ was randomly chosen from a glass surface with an AFM cantilever tip and stretched in PBS buffer containing 2 mM (2-carboxyethyl)phosphine (TCEP) in the absence of gold ions. Because the attaching points of the polyprotein are uncontrolled, the number of protein domains being unfolded varies in different traces. Stretching (beta GB1-GolB)$_4$ results in force-extension curves that are characterized by a featureless long spacer followed by the characteristic unfolding peaks of GB1 (unfolding forces ~180 pN and ΔLc of ~18 nm)[27,29,30]. Because GolB alternates with GB1 in the polyprotein, the long, featureless spacer preceding GB1 unfolding events must result from the stretching and unfolding of GolB. Such long, featureless spacers indicate that GolB unfolds at forces that are below the detection limit

of our AFM (~10 pN). The distributions of the unfolding forces and contour length increments for GB1 are shown in Figures 2B and 2C, respectively. Then, we stretched the same polyprotein charged with gold ions. In addition to the presence of unfolding events of GB1, we observed the force peaks with ΔLc value of ~9 nm (Figure 2D). Because the beta hairpin inserted into GolB has 15 amino acids, corresponding to a contour length of ~ 9 nm (0.365 nm/aa *(15+4) aa = 6.9 nm), we conclude that these force peaks resulted from the rupture of Au-S bonds in GolB. The ratio between the events from the rupture of Au-S bonds and those from GB1 unfolding suggest that ~ 50% of GolB was bound with gold ions under experimental conditions (Figures 2E and 2F). Surprisingly, we found that the forces of these peaks are similar to the unfolding forces of GB1, indicating that the mechanical stability of Au-S bonds in GolB is low for a coordination bond. The distribution of the rupture forces of the Au-S bonds in GolB is shown in Figure 2E. The average forces are 165±55 pN, which are significantly lower than the mechanical stability of Au-S bonds from other non-protein gold complexes (~ 0.5-1 nN)[8]. Interestingly, the rupture forces are similar to those of Fe-S bonds in rubredoxin[24]. This provides additional evidence that the strength of the coordinate bonds can be dramatically modulated by the protein environment. We further studied the Cu-S bond in GolB when the protein is charged by copper ions. Similarly, we observed force peaks with contour length increments of ~9 nm for the rupture of Cu-S bonds in addition to the unfolding events for GB1 (Figure 2G). Interestingly, the rupture forces for Cu-S are comparable with those for the Au-S bond in GolB, while there is significant difference in binding affinity between GolB to Au and Cu[16]. Notably, the observed rupture events for Cu-S bonds are around 52% of the unfolding events for GB1 (Figures 2H and 2I), indicating that the Cu-S bonds in GolB are comparable with the Au-S bonds (50%). The bond strength for Au-S and Cu-S is much less than that for disulphide bonds with rupture forces more than 450 pN under the same condition (Figure S2)[31]. Therefore, we hypothesize that the protein environment of GolB plays a major role in modulating the mechanical stability of the metal-thiol bonds.

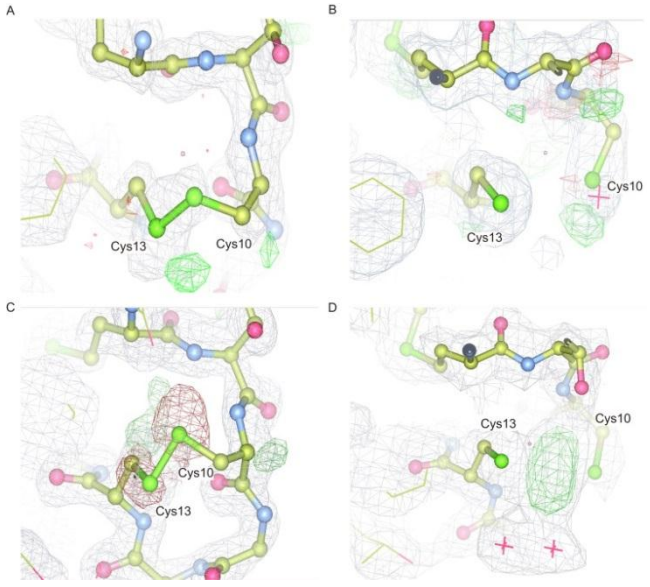

Figure 3. Stereo view of 2Fo-Fc electron density map (light grey, contoured at 1.00σ) at the metal binding site in the GolB X-ray structure. (A) The Au(I)-soaked structure. (B) The TCEP-soaked structure. (C) The negative density demonstration in the TCEP-soaked structure. (D) The Au(I)- and TCEP-soaked structures (green, contoured at 9σ).

To further understand the weak mechanical strength of the Au-S bond in GolB at the atomic level, we solved the crystal structures of GolB proteins in different states, including oxidized/reduced GolB and Au(I)-GolB complex. First, gold ions were used as the heavy atom derivative to solve the phase by the Single Wavelength Anomalous Dispersion (SAD) method and the oxidized apo-GolB structure was obtained at a 1.40 Å resolution. The structure of oxidized apo-GolB showed that the two cysteines (Cys^{10} and Cys^{13}) in the CXXC domain formed a disulfide bond without Au(I) binding (Figure 3A), even in the heavy atom derivative structure. Next, treatment of the reductive TCEP resulted in a reduced apo-GolB structure with a 1.80 Å resolution where the disulphide bond between Cys^{10} and Cys^{13} was broken (Figure 3B). The negative density in Figure 3C confirmed that there was no disulfide bond formated between the two cysteines in the reduced form of apo-GolB. Last, in the sequential soaked structure of TCEP and Au(I), the gold atom was found between Cys^{10} and Cys^{13}, forming two coordination bonds as shown in Figure 3D.

At 1.80 Å resolution, the Cys10 Cα atom in the reduced form of apo-GolB moved away from its position in the oxidized

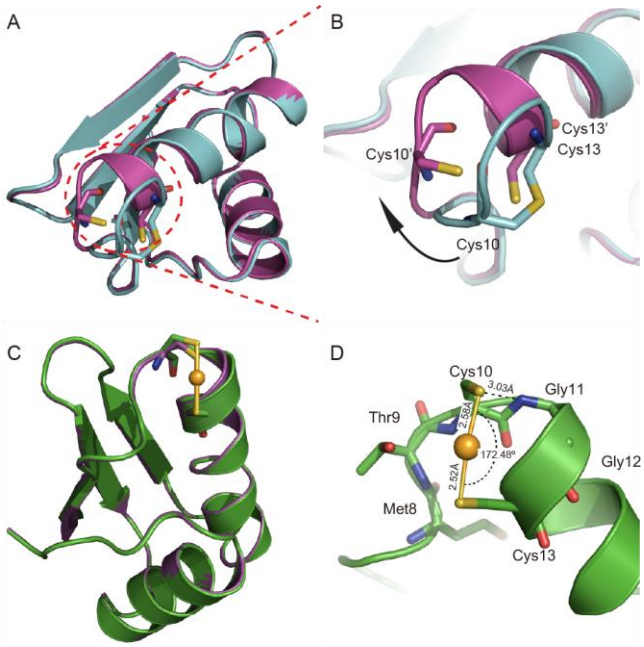

Figure 4. (A) Superposition of the overall structures of oxidized apo-GolB (cyan, PDB accession code: 4Y2M) and reduced apo-GolB (magenta, PDB accession code: 4Y2K). (B) Close-up view of the Cys10 shift between oxidized apo-GolB and reduced apo-GolB. (C) Superposition of the overall structures of reduced apo-GolB (magenta) and Au(I)-bound GolB (green). (D) Close-up view detailing the Au(I)-S coordinate-covalent bonds and hydrogen-bonding interactions at the GolB metal-binding pocket.

form, with a distance of 4.79 Å. In contrast, the Cys13 Cα atom stayed in the rigid helix area (α1), most likely to open an appropriate space for metal ions participating in a disulfide bond with Cys[13] (Figure 4A,B). This shift changes the position of the metal binding loop between residues 10 and 13, while the rest of the structure is unaltered. Furthermore, the structure of Au(I)-bound GolB was determined at 2.00 Å resolution (PDB accession code: 4Y2I), with a measured distance of two sulfur atoms at 5.09 Å. Compared to the reduced apo-GolB structure, it shows little conformational changes except the metal binding domain(Figure 4C). The coordinate-covalent Au-S bonds reveal a bond angle of 172.48º, which is similar to reported S-Au(I)-S linear coordination[32] (Figure 4D). The average Au-S bond length (2.55 Å) is much longer than the reported average value (~2.40 Å)[13,33]. It is worth noting that because the Au binding motif is located on the N-terminus of an alpha helix, the two Au-binding thiolates are surrounded by positively charged backbone NH groups. The favorable interaction between the cysteines with helix dipole could greatly lower the coordination capability of the sulfide. Especially, the backbone NH from amino acid Gly[11] is only 3.03 Å away from Cys[10] thiolate (Figure 4D), which could potentially form hydrogen bonds with the thiolate. The charge-charge interaction and hydrogen bonding among them could greatly neutralize the charge of thiolate and thus weaken the Au-S bonds[13].

The structure-property relationship of a chemical bond is of fundamental importance in science. However, most studies have focused mainly on chemical bonds in simple chemical environments. Here we show that in single-molecule settings, the properties of a chemical bond can be significantly modulated. The generally believed highly covalent and strong Au-S bonds are much weaker in a protein than those on a non-protein surface. The rupture forces for the Au-S bonds are only ~165 pN, much lower than the Au-S bonds measured on different gold surfaces[6-8]. It is worth mentioning that the chemical environment on gold surfaces is poor understood. This could lead to dramatically different mechanical strength of Au-S bonds.[8] Currently, it is still technically difficult to study the mechanical strength of Au-S bonds in well-defined inorganic complexes. Although the protein environment is also complex, it is well defined and can be further understood through X-ray crystallography. Therefore, protein system provides us tremendous possibilities to understand the fundamental metal-thiol binding in details. The mechanical stability of Au-S bond in GolB is comparable to many non-covalent interactions, including the rupture of streptavidin-biotin interaction and protein unfolding. Similarly, Zheng and Li demonstrated that the Fe-S and Zn-S bonds that of high covalency in a protein environment are also mechanically weak (**Table 1**)[24,34]. Moreover, two inspiring reports from Garcia-Manyes group respectively showed that the mechanical lability of the individual Zn-S bonds in zinc finger was only ~90 pN, and the rupture forces of Cu-S bonds in plastocyanin and azurin are only ~45 pN[15,35]. The strength of the metal coordination bonds could be precisely tailored in protein environments through evolution for specific biological functions. The combination of X-ray crystallography and SMFS provides a unique way to probe the underlying mechanism.

Table 1: Strengths of single metal- thiol bonds in protein or non-protein surfaces

Single thiol-metal bond	Strength（pN）		Reference
	Protein	Non-protein	
Au-S	165±55		This work
		1400±300	Ref.6
		2200~2900	Ref.7
		500~1000	Ref.8
Cu-S	171±47	NA	This work
	~45		Ref.33
Zn-S	~170	NA	Ref.31
	~90		Ref.32
Fe-S	~211	300~500	Ref.23

Based on our results, the low mechanical stability of Au-S bonds in GolB may originate from the following two major aspects. First, the low mechanical stability of Au-S bonds in GolB might be associated with the chemical environment around the Au-S bonds in the protein. As revealed by the crystal structure, GolB exhibits a classic βαββαβ fold structure similar to that observed for other homologues[36,37]. Two Au-binding thiolates from Cys[10] and Cys[13] are surrounded by backbone NH groups of residues 8-13 in a range of approximately 3-4 Å. As well documented in literature[38,39], such a positively charged environment around the N terminus of a helix dipole could potentially neutralize the charges on the thiolates by direct charge-charge interactions. Moreover, the hydrogen bonding could further weaken the chelation capability of the thiolates, leading to weakened Au-S bonds.[13] Second, the length of Au-S bonds in GolB is much longer than those in inorganic complexes; hence, the Au-S bond in GolB bond is intrinsically weaker.

Our finding that the Au-S bonds in GolB are mechanically weak may be valuable for the understanding of the biological function of GolB protein. According to previous research on the gold sensing and resistance function of *Salmonella gol* regulons, GolB protein expression is regulated by GolS under gold ion induction[19]. It was proposed that GolB needs to competitively bind toxic gold ions with a high affinity, while not affecting the function of other copper trafficking proteins. Additionally, GolB also must deliver gold ions to the P-type ATPase GolT, which functions as gold transporter, similarly to the reported function of copper chaperones for copper trafficking. Thus, the combination of high gold binding affinity and mechanically weak Au-S bonds allows GolB to robustly sustain the biological function as a gold chaperone, which might suggest a general principle for the trafficking of metal ions *in vivo*.

ASSOCIATED CONTENT

Supporting Information

Experimental details and crystallographic data. The structure has been deposited in the Protein Data Bank as entry 4Y2M,

4Y2K, 4Y2I. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

ACKNOWLEDGMENT

Financial support was provided by the Doctoral Fund of the Ministry of Education of China, the Guangdong Government (S20120011226), the National Science Foundation of China (21332005, 21571098 and 31200607) and the MOST of China (2014AA020512).

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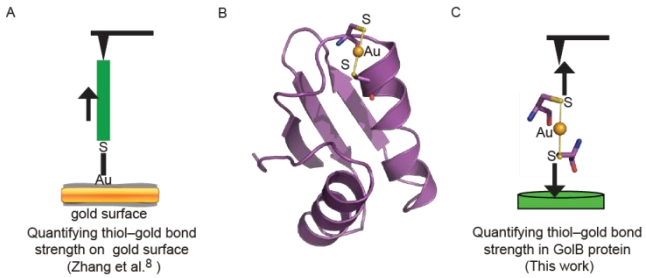

Keywords: Au-S bond, mechanical stability, metalloprotein, bond strength, gold