BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

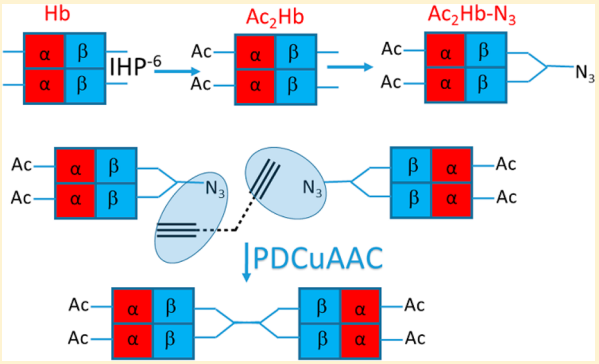
pubs.acs.org/biochemistry

Increasing Efficiency in Protein–Protein Coupling: Subunit-Directed Acetylation and Phase-Directed CuAAC ("Click Coupling") in the Formation of Hemoglobin Bis-Tetramers

Aizhou Wang and Ronald Kluger*

Davenport Chemical Laboratories, Department of Chemistry, University of Toronto, Toronto, Ontario, Canada M5S 3H6

ABSTRACT: Cross-linked human hemoglobins have been evaluated for clinical use as circulating oxygen carriers. However, their induction of vasoactivity was sufficiently problematic to lead to the cessation of clinical trials. The source of vasoactivity is likely to be endothelial extravasation causing the scavenging of endogenous nitric oxide. It was recently shown that species that consist of two coupled hemoglobin tetramers do not evoke vasoactivity in a sensitive murine model. Presumably these materials are too large to extravasate. In order to make this class of material more readily available, there is a need for improved methods that can form a cross-linked bis-tetramer without producing smaller species at the same time. A potentially efficient route to cross-linking and coupling two Hb tetramers is through phase-directed copper-catalyzed azide alkyne cycloaddition (PDCuAAC). However, introduction of the necessary azide-containing cross-link gives mixtures of tetrameric and bis-tetrameric proteins, as the PDCuAAC process appears to be limited to only those proteins where the cross-link containing the azide is exclusively within the β-subunits. In order to block formation of the azide cross-link within the α-subunits, subunit-specific introduction of the azide is necessary. This is achieved by blocking reaction at the reactive amino groups of the β-subunits in the site that binds the allosteric activator 2,3-diphosphoglycerate (DPG) with inositol hexaphosphate (IHP), permitting α-selective acetylation with acetyl 3,5-dibromosalicylate. After removal of IHP, reaction with an anionic cross-linker containing an azide group occurs within the β-subunits. The resulting α-acetylated β–β'-cross-linked hemoglobin azide (acHb>-N_3) undergoes efficient PDCuAAC with bis-alkynes to produce cross-linked bis-tetramers. Analysis of circular dichroism spectra of the modified species shows that there is little change in the structure of the globin chains as a result of the chemical modifications. The oxygenation properties are consistent with those needed for effective oxygenation in circulation, while the bis-tetrameric structure is sufficiently large to avoid extravasation and depletion of nitric oxide.



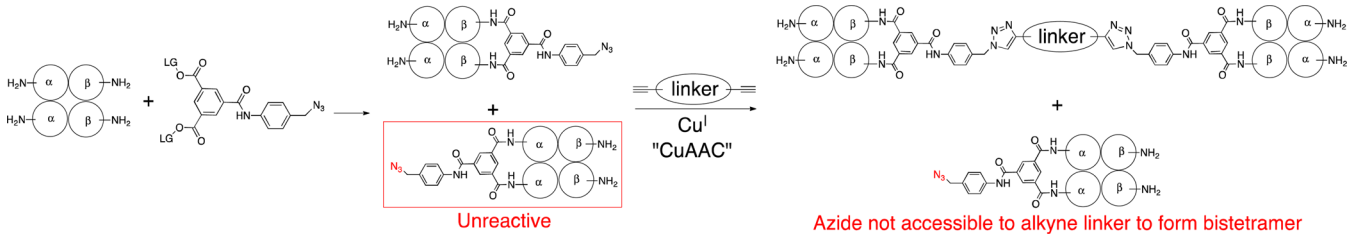
The potential utility of an acellular oxygen-carrier as an alternative to red cells in transfusions led to the design and production of various chemically altered derivatives of human hemoglobin (Hb).[1] In acellular circulation, the native protein dissociates from a functional tetrameric ($\alpha_2\beta_2$) state into nonfunctional $\alpha\beta$ dimers. Introduction of chemical cross-links within Hb permanently stabilizes the tetrameric form while producing desirable oxygenation properties. Reports of clinical evaluations of cross-linked tetramers in circulation revealed their tendency to induce significant hypertension.[2,3] The observed vasoactivity is likely to be associated with extravasation of the tetramers into the endothelia and scavenging of nitric oxide, which signals relaxation of the blood vessels.[4] Constricted vessels would be expected to lead to cardiac stress. The observation of these side effects led to termination of clinical trials for potential HBOCs.[5] On the basis of this understanding, an acceptable design for an oxygen carrier from hemoglobin must overcome extravasation as well as transport oxygen. It is possible that increasing the size of the cross-linked Hb derivative should resist extravasation. It appears likely that connecting two hemoglobins would produce a species that would not extravasate. The initial route to a cross-linked bis-tetramer utilized tetrafunctional reagents that consist of linked pairs of conventional site-directed cross-linking reagents.[6] The resulting bis-tetramers bind oxygen reversibly with appropriate affinity and cooperativity. Zapol and co-workers[7] evaluated one example in circulation in mice whose condition is critically dependent on the availability of nitric oxide. They noted that in accord with expectations the materials did not evoke symptoms associated with vasoactivity. On the basis of those positive observations with a highly purified cross-linked bis-tetramer, the general approach seems promising, unlike alternatives that are mixtures from less specific approaches that can also contain unlinked hemoglobin α- and β-subunits, $\alpha\beta$-dimers, and single tetramers, cross-linked or not.

The approach used to produce the tested bis-tetramer from tetra-esters is affected by competing spontaneous hydrolysis,

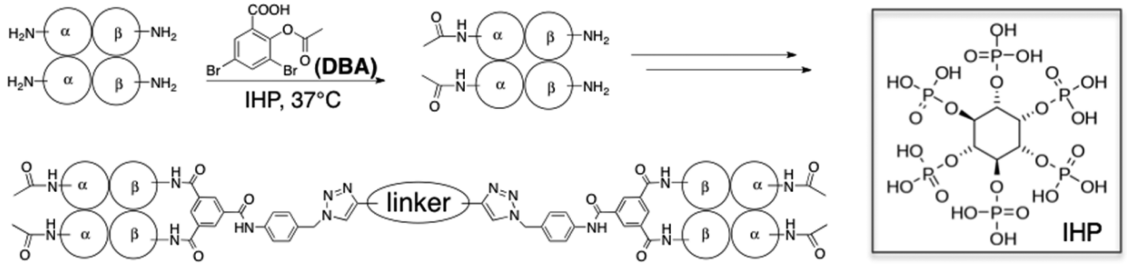
Received: August 21, 2014
Revised: October 2, 2014
Published: October 7, 2014

dx.doi.org/10.1021/bi501054b | *Biochemistry* 2014, 53, 6793–6799

Scheme 1. Generation of a Hemoglobin Bis-Tetramer Using an Azide-Functionalized Cross-Linker



Scheme 2. Blocking α-Lys-99 Amino Site via Acylation Eliminates Undesired Cross-Linked Tetramer



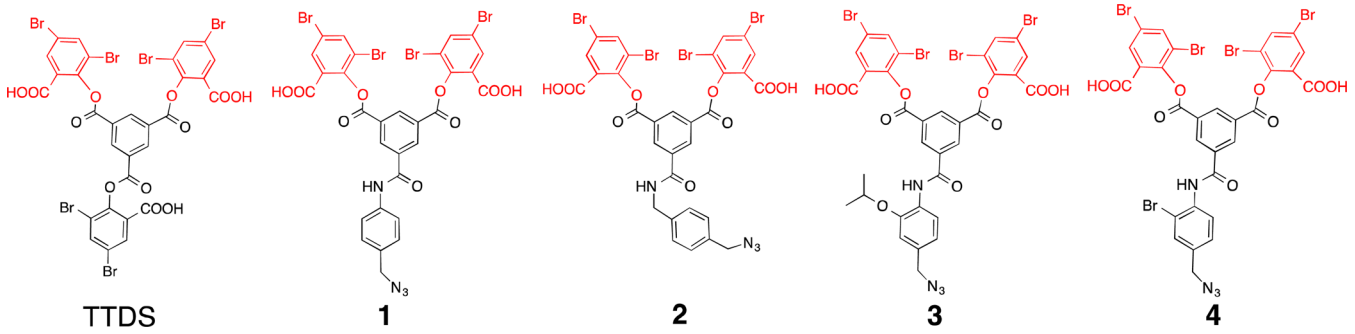


Figure 1. Azide-functionalized cross-linkers (**1**–**4**) that are designed based on a β-specific cross-linker trimesoyl tris(3,5-dibromosalicylate) (TTDS); all the linkers are activated by dibromosalicylyl (DBS) group, as shown in red.

rendering those hydrolyzed sites unreactive toward the protein. The residual sites leave unconnected dimers as well as partially modified tetramers. Separation of the components of the product mixtures to isolate pure bis-tetramers is technically challenging and inefficient. In order to overcome the problems associated with hydrolysis in the one-step approach to formation of bis-tetramers, we devised an alternative approach.[8] First, a cross-link containing an azide is produced in each tetramer. The azides then undergo pairwise cycloaddition to a bis-alkyne in the presence of Cu(I), utilizing the process with the acronym CuAAC that is phase-directed (PDCuAAC) so that the bis-alkyne is preferentially modified at both ends.[9]

We observed that where the cross-link containing the azide is between β-Lys-82 and β'-Lys-82, the PDCuAAC process occurs as expected. However, the azide-containing anionic cross-linker that produces the $\beta\beta$ cross-link also produces a link between α-Lys-99 and α'-Lys-99. The latter product does not undergo the CuAAC process at all, suggesting that the azide is not accessible to react with an alkyne probably because it is surrounded by substantial portions of the protein (Scheme 1).

Our assessment of site-selective cross-linking reagents containing the azide functional group revealed that they inevitably acylate amino groups in the α-subunits as well as the desired ones in the β-subunits (Y. Yang, personal communication). Therefore, in order to direct the azide-containing cross-linker to react only with amino groups in the β-subunits, we developed a general approach by first chemically blocking the α-subunit's groups using 3,5-dibromoacetyl salicylates to acetylate those amino groups so that they would not react with the cross-linker. This is followed by the cross-linking reaction, which is then directed to the reactive amino groups in the β-subunits at Lys-82 (Scheme 2).

In order to direct acetyl 3,5-dibromosalicylate to react in the α-subunits, we first add inositol hexaphosphate (IHP), a polyanion that has previously been shown to bind non-covalently in a highly cationic region that is principally associated with the β-subunits and normally binds 2,3-diphosphoglycerate (DPG), an allosteric effector for oxygen binding. Removal of IHP follows the α-acetylation reaction opening the DPG-binding site to introduction of the β–β cross-linker into the α-acetylated Hb. By this route, the resulting β–β'-cross-linked hemoglobin azide is produced with greater efficiency than when the reaction is run without first blocking the α-subunits. The resulting bis-tetramer is then formed with improved efficiency by PDCuAAC.

MATERIALS AND METHODS

All reagents were obtained from commercial suppliers and used without further purification unless otherwise indicated. Highly purified human hemoglobin A (Hb) was obtained from Oxygenix Inc. Concentrations of hemoglobin solutions were determined using the cyanomethemoglobin assay.[10] The purity

dx.doi.org/10.1021/bi501054b | *Biochemistry* 2014, 53, 6793–6799

of hemoglobin was determined through reverse-phase HPLC analysis as described by Jones.[11] Trimesoyl tris(3,5-dibromosalicylate) (TTDS) and azide-functionalized cross-linkers (Figure 1) were used as previously prepared stock. 3,5-Dibromoaspirin (DBA) was synthesized as described previously:[12] ^{1}H NMR (399 MHz, DMSO-d_6) δ 8.26 (d, J = 2.4 Hz, 1H), 8.02 (d, J = 2.4 Hz, 1H), 2.31 (s, 3H).

Preparation of Acetylated Hemoglobin (acHb). Stock carbomonoxy-hemoglobin (HbCO) (1.0 mL, 1.5 mM) was passed through a Sephadex G-25 column equilibrated with bis-Tris buffer (0.2 M, pH 7.2). The resulting solution was photoirradiated at 0 °C under a stream of humidified oxygen for 2 h to give oxy-hemoglobin (HbO_2). IHP (4 equiv) was added and the mixture was deoxygenated under a stream of humidified nitrogen at 37 °C for 2 h to give deoxy-hemoglobin (deoxyHb). DBA (4 equiv) was added as a solid with minimal exposure of the deoxyHb solution to air. The reaction was allowed to proceed for 20 h under a stream of humidified nitrogen at 37 °C. Upon reaction completion, the mixture was placed under a stream of humidified carbon monoxide for 15 min, filtered with a 0.45 μm syringe filter, and passed through a Sephadex G-25 column equilibrated with MOPS buffer (0.1 M, pH 8). The resulting modified HbCO was concentrated to ~1.0 mL, sealed in an Agilent vial, and stored at 4 °C.

Testing Reactivity of β-Lys82 of acHb: Preparation of xl-acHb Using TTDS. acHb (1.0 mL, 1.03 mM) was passed through a Sephadex G-25 column equilibrated with sodium borate buffer (0.05 M, pH 9.0). The acHbCO solution was oxygenated and deoxygenated to give deoxy-acHb. TTDS (3 equiv) was added as a solid with minimal exposure of the deoxy-acHb solution to air. The reaction and workup procedures are similar to those described above for Hb acetylation.

HPLC Analysis of Modified Hemoglobin. Modified hemoglobin was analyzed using analytical reverse-phase HPLC with a 330 Å C-4 Vydac column (4.6 × 250 mm) to determine the sites of globin chain modifications.[13] Modified and unmodified globin chains were separated using an eluting solvent containing 0.1% trifluoroacetic acid and a gradient beginning with 20% and ending with 60% acetonitrile (vol %) in water. The effluent was monitored at 220 nm. The cross-linked tetramers and bis-tetramers were analyzed using a Superdex G-200 HR (10 × 300 mm) preparative size exclusion column. Protein samples were eluted under high salt conditions that dissociate the native hemoglobin tetramers into $\alpha\beta$ dimers (37.5 mM Tris-HCl, pH 7.4, 0.5 M magnesium chloride). The effluent was monitored at 280 nm.

SDS-PAGE Analysis. The molecular weights of constituent proteins were estimated using polyacrylamide gel (12% Tris-HCl) electrophoresis. Two-dimensional Tris-HCl polyacrylamide gels were comprised of 12% separating gel (pH 8.8) and 5% stacking gel (pH 6.8) both with 10% sodium dodecyl sulfate. Protein samples were treated with 2-mercaptoethanol and sodium dodecyl sulfate.[12] Globin chains were further denatured by heating at 95 °C for 15 min before being loaded onto the gel. Finished gels were stained with Coomassie Brilliant Blue. Further details are described in earlier papers.[11]

Preparation of Cross-Linked acHb>-N_3 Using Azide Functionalized Cross-Linker 4. acHb (7.5 mL, 0.44 mM) was passed through a Sephadex G-25 column equilibrated with sodium borate buffer (0.05 M, pH 9.0). The procedures for cross-linking reactions were similar to those described for xl-acHb production. In this case, the cross-linker **4** (0.2 mL, 124 mg/mL in DMSO) was added to the reaction mixture. The DMSO volume was kept around 1% of the final solution.

Heat Treatment. The protein sample concentration was diluted to ~0.05 mM with MOPS (0.1 M, pH 7.2) and sealed in a vial with a mini stir bar. The sample was flushed with carbon monoxide for 15 min, then placed in a hot water bath at 75 °C and stirred for 30 min. After the heat treatment, the solution was transferred to an ice-bath and flushed with carbon monoxide for 10 min. Precipitated protein was removed via a 0.45 μm syringe filter. The filtrate was passed through a Sephadex-G25 column equilibrated with MOPS buffer (0.1 M, pH 8), concentrated, flushed with CO, and stored at 4 °C.

Oxygen Binding Analysis. Oxygen binding measurements (28 °C, pH 7.4) were conducted with a Hemox analyzer, which measures the partial pressure of oxygen (P_{O_2}) and the fractional saturation of oxygenated hemoglobin (Y). Hemoglobin samples (~1.0 g/L), in sodium phosphate buffer (I = 0.01 M, pH 7.4) were photoirradiated under a stream of humidified oxygen before analysis. The oxygenated samples were then deoxygenated by streaming nitrogen. The Hemox analyzer follows the deoxygenation process until the P_{O_2} reaches a minimum value. The data were then fit to the Adair equation[15] to obtain the half-saturation pressure (P_{50}) and Hill's coefficient of cooperativity at half saturation (n_{50}).

Circular Dichroism. Native hemoglobin (Hb) and modified hemoglobin (acHb and acHb>-N_3) in CO-bound form were studied by circular dichroism spectrometry in the far ultraviolet regions (200−260 nm) at room temperature.[11] Hemoglobin samples were prepared as 10 mL solutions of the same globin chain (heme) concentration in the sodium phosphate buffer (0.02 M, pH = 7.4). The globin chain (heme) concentration of the hemoglobin samples was 5 μM.

Reaction of acHb>-N_3 with a Bis-Alkyne. The CO-bound form of acHb>-N_3 (1 mL, 1.45 mM) was passed through a Sephadex G-25 column equilibrated with phosphate buffer (0.02 M, pH 7.4). The volume of the resulting solution was adjusted to 4 mL and transferred to a reaction vial. The bis-alkyne was then added (145 μL, 0.1 M in DMSO) along with batho ligand (290 μL, 20 mM in water), $CuSO_4$ (145 μL, 20 mM in water), L-ascorbic acid (580 μL, 0.1 M in water), and a stirring bar. The vial was sealed and the mixture was stirred at room temperature. The reaction was quenched after 5 h via filtration of the insoluble bis-alkyne through a 0.45 μm syringe filter. The filtrate was passed through a Sephadex G25 column that had been equilibrated with MOPS buffer (0.1 M, pH 7.2) twice and centrifuged to remove any residual copper salts. The resulting product was concentrated and stored at 4 °C.

RESULTS AND DISCUSSION

Acetylation of Amino Groups in the α-Subunits of HbA. IHP specifically associates with the β-subunits of Hb in the cationic site that normally binds 2,3-DPG. Klotz, Walder and co-workers used this association to direct an anionic cross-linker away from reaction at the β-subunits and into the α-subunits at Lys-99.[15,16] We find that consistent with those observations, reaction of Hb with acetyl 3,5-dibromosalicylate in a solution containing IHP produces acetylation of the accessible α-99 side chain amino groups of Hb based on analysis by reverse phase C4-HPLC. Under conditions that produce complete dissociation of the subunits, the modified and unmodified globin chains are separated, revealing

dx.doi.org/10.1021/bi501054b | Biochemistry 2014, 53, 6793−6799

information that permits identification of the sites that have become modified.

Each peak from the C4-HPLC of acHb (Figure 2) was isolated, and the mass of each peak was measured using ESI-MS

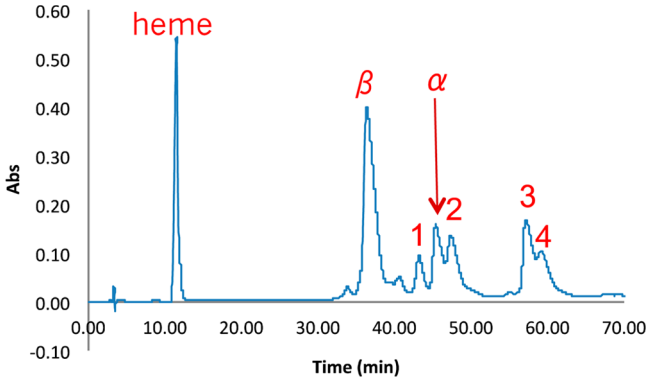


Figure 2. rpC4-HPLC analysis for the formation of acHb.

(+). Unmodified α, β, and heme peaks were identified by overlaying the chromatogram with that of the coinjected native hemoglobin. The mass results are summarized in Table 1. Over

Table 1. Mass Analysis of Peaks Isolated from C4-HPLC of AcHb (Figure 1)

peak	mass calculated (Da)	mass found (Da)	species
α	15867		Hbα
β	15126		Hbβ
1	15867	15867	Hbβ-ac[a]
2	15168	15168	Hbα-ac[a]
3	15168	15168	Hbα-ac[a]
4	15210	15210	Hbα-$(ac)_2$[b]

[a]Hbβ-ac and Hbα-ac correspond to monoacetylated hemoglobin β and α globin chains, respectively. [b]Hbα-$(ac)_2$ correspond to bis-acetylated hemoglobin α globin chain.

70% of α subunits are modified: peaks 2 and 3 correspond to the monoacetylated α-subunits. Peak 4 corresponds to the bis-acetylated α-subunits. Since reverse phase chromatographic separation is based on polarity, the differential retention times of peaks 2 and 3 probably correspond to α subunits that are acetylated at different locations that lead to differing effects on polarity.

We demonstrated that IHP effectively blocks acetylation of the amino groups in the DPG-binding site of the β subunits by acetyl 3,5-dibromosalicylate. With those sites blocked, the reagent can acetylate other amino groups at a low level within the β-subunits as well as the more reactive α-Lys 99 side chain amino groups. The presence of an acetylated amino group in the β-subunits is indicated by the presence of peak "1" in Figure 2. We tested the availability of the ε-amino groups of β-Lys 82 by reaction with trimesoyl tris-dibromosailicylate (TTDS), a reagent that reacts selectively with the ε-amino groups of β-Lys82 of deoxyHb.[17] We compared the chromatograms from the products of the reaction of TTDS with Hb (xl-acHb) and the acetylated derivatives (acHb). C4-HPLC analysis of the product from the reaction of β-acHb with TTDS shows no peak corresponding to β-acetylated derivatives (β-ac, Figure 3a) and instead shows the same pattern as the β-82 cross-linked species derived from native Hb. This establishes the availability of β-Lys82 of acHb. Thus, in the presence of IHP, the amino groups at each β-Lys-82 are not modified by acetyl 3,5-dibromosalicylate.

This was further confirmed using Superdex G200 size-exclusion chromatographic analysis. Placing Hb that is not cross-linked in high salt solutions causes that tetramer to dissociate into $\alpha\beta$-dimers. The resulting chromatograms (Figure 3b) show that under these conditions, acHb undergoes dissociation into dimers, while the product from its further reaction with TTDS (xl-acHb) gives no peak corresponding to the dimers.

We find that Hb is acetylated most efficiently in the deoxy state (Table 2). This is consistent with the central cavity being more accessible when Hb is deoxygenated. The optimal conditions for acetylation of the amino groups of α-Lys-99 were determined by examining the pH-dependence of the acetylation process (Figure 4). In principle, acetylation will be more rapid at higher pH due to the increased availability of amino groups of lysine residues that are not protonated. However, we find that when the pH of the reaction solution is increased from 7.2 to 9.0, the preferred subunit for acetylation shifts from α to β. This shift in specificity is likely due to the reduction in the amount of unsolvated net positive charge of

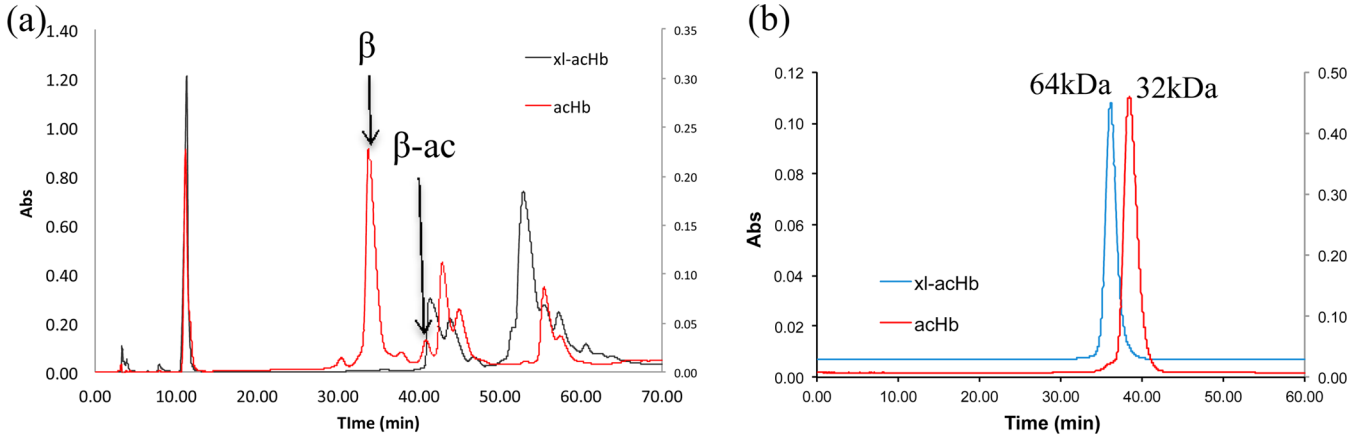


Figure 3. (a) rpC4-HPLC chromatography of the formation of xl-acHb from acHb using β-specific cross-linker TTDS. Disappearance of the β-ac peak after reaction with TTDS indicates that the initial site of acetylation in the β is not β-Lys82 of t. (b) Size exclusion G200 HPLC of the xl-acHb indicates that acHb (32 kDa) is turned into completely cross-linked tetramer (64 kDa).

dx.doi.org/10.1021/bi501054b | *Biochemistry* 2014, 53, 6793−6799

Table 2. Acetylation of Hemoglobin under Different Conditions

buffer system	Hb	DBA (equiv)	IHP (equiv)	rxn length (hr)	% β modified[a]	% α modified[a]
MOPS (0.1 M, pH 8)	CO	3	5	20	1	19
	deoxy	4	5	20	44	56
bis Tris (0.2 M, pH 7.2)	deoxy	4	5	20	3	64
		8	16	20	5	77
		12	5	5	16	80
sodium borate (0.05 M, pH 9.0)	deoxy	4	5	20	72	69

[a]%mModification is calculated by the formula (area(peak modified))/(area(peak modified + native)) with the assumption that absorption coefficient of each subunit does not change upon modification.

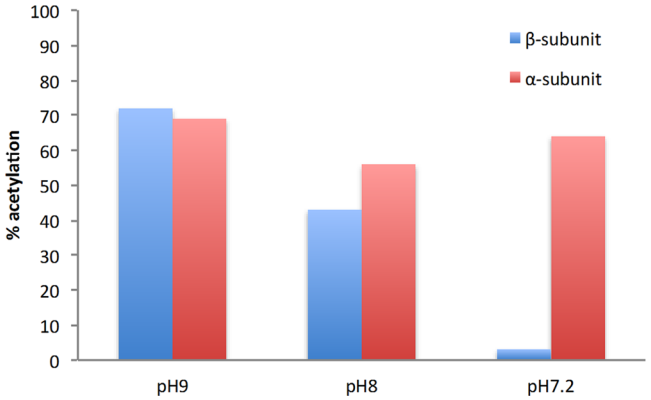


Figure 4. Subunit selectivity of acetylation under different buffer pH with all other reaction parameters fixed: deoxy Hb, 4 equiv of DBA, 5 equiv of IHP, and 20 h reaction length.

the DPG site in the β cleft, which is normally highly cationic in neutral solution.

Preparation of Cross-Linked acHb>-N_3 Using an Azide Functionalized Cross-Linker and Heat Treatment. The reaction of cross-linker **4** with deoxy-Hb (native Hb and acHb respectively) was done to compare the effectiveness of the α-blocking in improving the β vs α site-selectivity for introduction of the azide-functionalized cross-linker. Analysis of the product by C4-HPLC under dissociating conditions showed that a singly linked β-azide-containing species (β-N_3), a cross-linked β-azide-containing species ($\beta\beta$>-N_3), and a cross-linked α-azide-containing species ($\alpha\alpha$>-N_3, Figure 5) form.

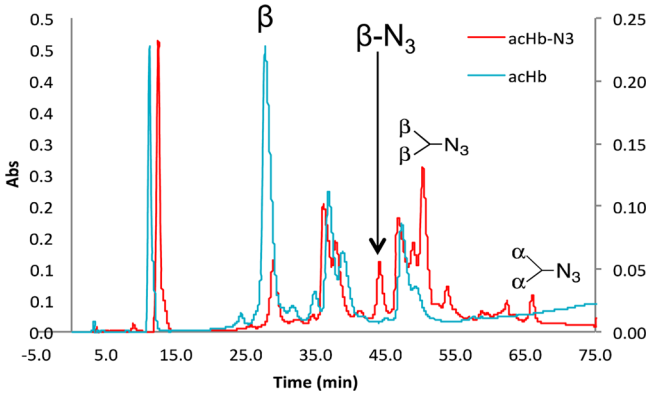


Figure 5. C4-rpHPLC analysis of the cross-linking reaction between acHb and **4**. The chromatogram of the product (acHb-N_3) is overlaid with the starting material (acHb): monolinked β-azide moiety (β-N_3), cross-linked β-azide moiety ($\beta\beta$>-N_3) and cross-linked α-azide moiety ($\alpha\alpha$>-N_3) were formed.

The yields for conversion of Hb and β-acHb are summarized in Table 3. The cross-linking reaction between native Hb and **4** has a 4:1 β:α selectivity. It is clear that the reaction of acHb gives a much higher β:α selectivity (15:1 vs 4:1), validating the acetylation process as an effective approach to directing cross-linking reactions to the β-subunits of Hb.

Table 3. Comparison of Native Hemoglobin Hb and α-Blocked acHb in β to α Selectivity for the Reaction with the Azide-Functionalized Cross-Linker 4

starting material	% β-N_3[a]	% $\beta\beta$>-N_3	% $\alpha\alpha$>-N_3	β:α
Hb	22	42	10	4:1
acHb	20	59	4	15:1

[a]Monolinked β-azide species with the other DBS group hydrolyzed during reaction.

In both cases, a monolinked species (β-N_3) is produced in about 20% of the materials. This corresponds to a β-subunit that reacts with one end of the linker **4** but is not able form a cross-link by reaction with another β subunit due to the hydrolysis of the other DBS group of the linker. The monomer-azide (β-N_3) is problematic due to its reactivity in CuAAC process as it will likely form a nonfunctional asymmetric dimer (β-link-β) with itself, as well as a trimer (β-link-$\beta\beta$) with the cross-linked $\beta\beta$>-N_3.

To purify the desired cross-linked tetramers, we took advantage of the enhanced thermal stability of cross-linked hemoglobins.[18] Heating solutions to 75 °C for 30 min denatures any species containing unreacted native β as well as the monomeric azide β-N_3. The efficiency of the heat treatment was assessed by analysis of the products using G200-HPLC (Figure 6). The disappearance of the 32 kDa shoulder in the G200 chromatogram indicates that all uncross-linked β

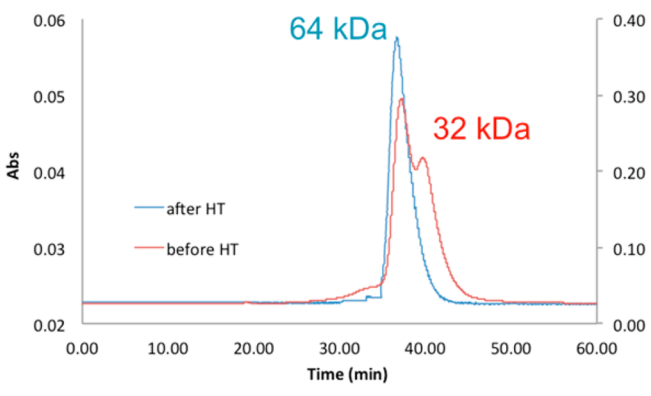


Figure 6. G200 HPLC analysis of the starting material (before HT) and product (after HT) of the heat treatment of acHb-N_3 mixture.

dx.doi.org/10.1021/bi501054b | Biochemistry 2014, 53, 6793−6799

moieties are denatured by this process. An incidental benefit is that this heat-treatment is equivalent to pasteurization, which is likely to inactivate bacteria and viruses.[19]

Oxygen Affinity and Cooperativity. The oxygen binding properties of acetylated hemoglobin derivatives were measured and compared with those of unmodified Hb (Table 4). By fitting the data to the Adair equation,[14] the oxygen dissociation curve was generated (Figure 7). The oxygen affinity (P_{50}) is derived at Y = 0.5. The oxygen affinity of acHb (P_{50} = 11.5 Torr) is about twice that of Hb (P_{50} = 5.7 Torr) at pH 7.4, while its cooperativity is maintained comparable to that of native Hb. On the other hand, the acetylated cross-linked bis-tetramer (P_{50} = 5.4 Torr) maintains native oxygen affinity and has a decreased Hill coefficient.

Table 4. Oxygen Binding Data for Modified Hbs[a]

sample	P_{50}	n_{50}
native	5.7 ± 0.2	3.0 ± 0.1
acHb	11.5 ± 0.3	2.9 ± 0.1
BT-acHb	5.4 ± 0.4	2.0 ± 0.1

[a]All measurements were conducted in triplicates.

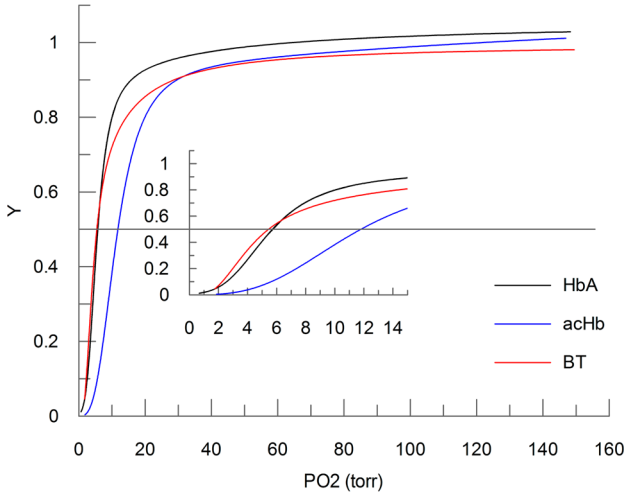


Figure 7. Oxygen dissociation curves of native hemoglobin (HbA), acetylated hemoglobin (acHb), and acetylated bis-tetramer (BT).

The 2-fold decrease in P_{50} is likely to be due to the introduction of the acetyl groups. The anionic acetylating agent targets hemoglobin's cationic DPG binding site. An over 2-fold decrease in oxygen affinity due to acetylation of α-amino groups in this site has been reported.[13] Those reports also note that such modifications do not have a significant effect on the protein's cooperativity in oxygen binding. The cooperativity is affected by the interprotein interactions of BT-acHb as the Hill coefficient dropped from 3 to 2. This reduction in cooperativity has also been observed in other bis-tetramers.[4]

Circular Dichroism. The CD spectra (Figure 8) of native hemoglobin and modified Hbs (acHb and acHb>-N_3) are nearly identical in the far UV region (220−260 nm), where the signals arise from the globin chain's structure. This shows that the introduction of an acetyl group into the protein probably does not significantly change the structure of the protein.

Reaction of acHb>-N_3 and a Bis-Alkyne To Afford the Bis-Tetramer of Hemoglobin (BT-acHb). Utilizing the

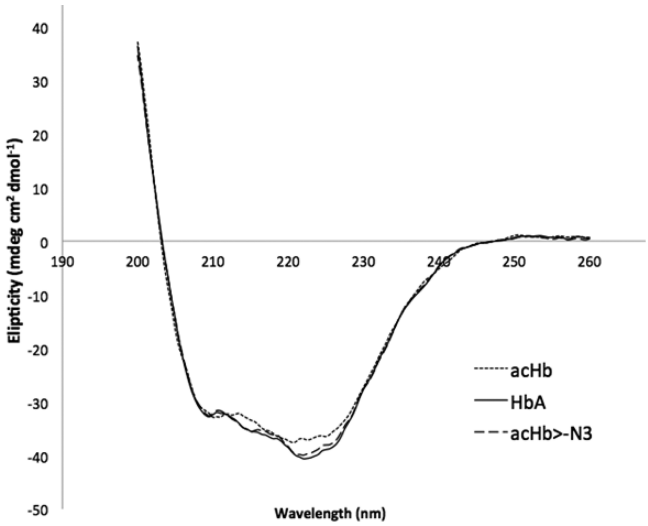


Figure 8. CD spectra of native hemoglobin (Hb), acetylated hemoglobin (acHb), and azide-functionalized cross-linked acHb (acHb>-N_3).

previously optimized conditions (for native hemoglobin azide),[10] the PDCuAAC reaction of heat-treated acHb>-N_3 with the bis-alkyne was carried out at room temperature in phosphate buffer (0.02 M, pH7.4) for 4 h. G200 HPLC analysis of the reaction mixture showed that acHb-N_3 converts over 65% of the protein to a bis-tetramer (Figure 9a), which is much higher than in cases without prior α-acetylation.

SDS-PAGE analysis (Figure 9) confirms the molecular weight of the modified Hb subunits in addition to gel filtration. The completely denaturing condition shows native monomeric globin chain for HbA at 16 kDa. The acetylated globin chain (lane "acHb") is similar to that of the native due to the small mass change. In the lane "xlHb", the higher mass band (32 kDa) corresponds to covalently cross-linked globin chain. PDCuAAC combines two acHb>-N_3 into a bis-tetramer that produces a band at 64 kDa (lane "BT crude"), indicating the covalently joined $\beta\beta$>-<$\beta\beta$ subunits. The unmodified cross-linked globin chain remains at 32 kDa and can be removed by gel filtration to produce pure bis-tetramer for characterization (lane "BT pure").

CONCLUSIONS

A high molecular weight cross-linked hemoglobin bis-tetramer (BT-Hb), produced by the PDCuAAC reaction between a bis-alkyne linker and azide-functionalized cross-linked hemoglobin, is an efficient coupling procedure to produce a bis-tetramer. However, all previously reported methods of introducing the β−β-cross-linked azide give heterogeneous outcomes, some of which do not lead to the desired bis-tetramer. In order to improve the efficiency of the bis-tetramer formation, the accessibility of the desired cross-linking site (β-Lys82) was strategically increased by making the undesired site (α-Lys-99) unreactive via site-selective acetylation with acetyl 3,5-dibromosalicylation in the presence of IHP. The high selectivity of α-subunit-blocking enhances the subsequent cross-linking reaction, in which a highly β-selective cross-linking result is obtained using acHb and cross-linker **4**. The resulting α-acetylated cross-linked hemoglobin azide (acHb>-N_3) produces a cross-linked bis-tetramer using the PDCuAAC method in a reaction with bis-alkynes. CD spectroscopy of the modified

dx.doi.org/10.1021/bi501054b | Biochemistry 2014, 53, 6793−6799

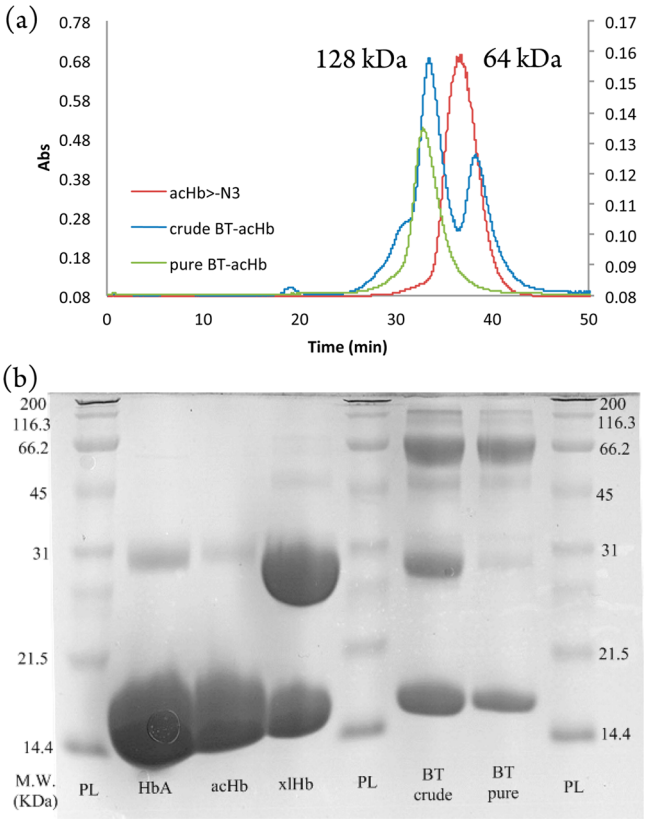


Figure 9. (a) G200-HPLC analysis of bis-tetramer (BT-acHb) formation from "click" of azide-functionalized cross-linked acHb (acHb>-N_3); (b) SDS-PAGE of native Hb and all the chemically modified Hb.

species shows that there is little change to the structure of the globin chains of Hb. Therefore, α-specific acetylation provides a means to introduce the necessary specificity for the cross-linking process that introduces an azide for the subsequent highly efficient reaction that joins two tetramers.

AUTHOR INFORMATION

Corresponding Author

*E-mail: r.kluger@utoronto.ca. Fax: 1-416-978-8775. Phone: 1-416-978-3582.

Funding

This work was supported by a grant from the Canadian Blood Service through the Canadian Institutes of Health Research. The training of A.W. is supported by a postgraduate scholarship from NSERC Canada and an Ontario Graduate Scholarship.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Ms. Megan Roberts provided helpful assistance throughout this project.

REFERENCES

(1) Elmer, J., Alam, H. B., and Wilcox, S. R. (2012) Hemoglobin-based oxygen carriers for hemorrhagic shock. *Resuscitation 83*, 285–292.

(2) Buehler, P. W., and Alayash, A. I. (2004) Toxicities of hemoglobin solutions: in search of in-vitro and in-vivo model systems. *Transfusion 44*, 1516–1530.

(3) Simoni, J., Simoni, G., and Moeller, J. F. (2009) Intrinsic toxicity of hemoglobin: how to counteract it. *Artif. Organs 33*, 100–109.

(4) Lui, F. E., and Kluger, R. (2009) Enhancing nitrite reductase activity of modified hemoglobin: bis-tetramers and their PEGylated derivatives. *Biochemistry 48*, 11912–11919.

(5) Natanson, C., Kern, S., Lurie, P., Banks, S., and Wolfe, S. (2008) Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death - A meta-analysis. *JAMA, J. Am. Med. Assoc. 299*, 2304–2312.

(6) Hu, D., and Kluger, R. (2008) Functional cross-linked hemoglobin bis-tetramers: geometry and cooperativity. *Biochemistry 47*, 12551–12561.

(7) Lui, F., Yu, B., Baron, D., Lei, C., Zapol, W., and Kluger, R. (2012) Hemodynamic responses to a hemoglobin bis-tetramer and its polyethylene glycol conjugate. *Transfusion 52*, 974–982.

(8) Foot, J., Lui, F., and Kluger, R. (2009) Hemoglobin bis-tetramers via cooperative azide-alkyne coupling. *Chem. Commun.*, 7315–7317.

(9) Yang, Y., and Kluger, R. (2010) Efficient CuAAC click formation of functional hemoglobin bis-tetramers. *Chem. Commun. 46*, 7557–7559.

(10) Drabkin, D., and Austin, J. (1935) Spectrophotometric studies II. Preparations from washed blood cells; nitric oxide hemoglobin and sulfhemoglobin. *J. Biol. Chem. 112*, 51–65.

(11) Jones, R. T. (1994) Structural characterization of modified hemoglobins. *Hemoglobins, Part B 231*, 322–343.

(12) Walder, J. A., Zaugg, R. H., Iwaoka, R. S., Watkin, W. G., and Klotz, I. M. (1977) Alternative aspirins as antisickling agents: acetyl-3,5-dibromosalicylic acid. *Proc. Natl. Acad. Sci. U.S.A. 74*, 5499–5503.

(13) Jones, R., Shih, D., Fujita, T., Song, Y., Xiao, H., Head, C., and Kluger, R. (1996) A doubly cross-linked human hemoglobin - Effects of cross-links between different subunits. *J. Biol. Chem. 271*, 675–680.

(14) Perutz, M. F. (1970) Stereochemistry of cooperative effects in haemoglobin. *Nature 228*, 726–739.

(15) Snyder, S. R., Welty, E. V., Walder, R. Y., Williams, L. A., and Walder, J. A. (1987) HbXL99 alpha: a hemoglobin derivative that is cross-linked between the alpha subunits is useful as a blood substitute. *Proc. Natl. Acad. Sci. U. S. A. 84*, 7280–7284.

(16) Wood, L. E., Haney, D. N., Patel, J. R., Clare, S. E., Shi, G. Y., King, L. C., and Klotz, I. M. (1981) Structural specificities in acylation of hemoglobin and sickle hemoglobin by diaspirins. *J. Biol. Chem. 256*, 7046–7052.

(17) Kluger, R., Song, Y. H., Wodzinska, J., Head, C., Fujita, T. S., and Jones, R. T. (1992) Trimesoyltris(3,5-dibromosalicylate) - Specificity of reactions of a trifunctional acylating agent with hemoglobin. *J. Am. Chem. Soc. 114*, 9275–9279.

(18) Kluger, R., Wodzinska, J., Jones, R. T., Head, C., Fujita, T. S., and Shih, D. T. (1992) Three-point cross-linking: potential red cell substitutes from the reaction of trimesoyl tris(methyl phosphate) with hemoglobin. *Biochemistry 31*, 7551–7559.

(19) Estep, T. N., Bechtel, M. K., Miller, T. J., and Bagdasarian, A. (1988) Virus inactivation in hemoglobin solutions by heat. *Biomater. Artif. Cells Artif. Organs 16*, 129–134.