Changing a Protein into a Generalized Acylating Reagent. Reaction of Nucleophiles with 3,5-Dibromosalicyl Trimesyl-((Lys-β-82)-(Lys-β-82))-Hemoglobin

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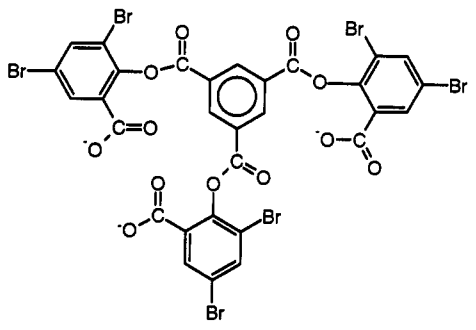
The reaction of human hemoglobin A with trimesoyl tris(3,5-dibromosalicylate) produces the 3,5-dibromosalicyl ester of the bis(amide) of trimesic acid and the ε-amino groups of each of the Lys-82 residues of the two β-subunits of hemoglobin (3,5-dibromosalicyl trimesyl-((Lys-β-82)-(Lys-β-82))-hemoglobin; DBST-Hb). This cross-linked protein containing a reactive ester site behaves as an acylating agent toward a variety of biochemically interesting nucleophiles. The products are acyl derivatives, as established by chromatographic and mass spectral analysis. The procedures demonstrate that the multifunctional reagent converts the protein into a reagent which is capable of directly generating bioconjugates of defined structure.

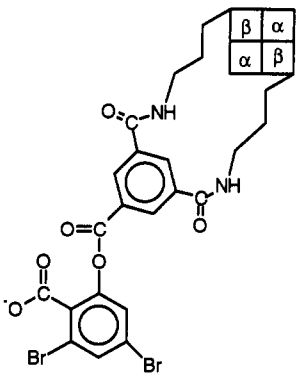
Site-specific chemical modification of proteins provides a potential route to novel materials which go beyond the limits imposed by the use of methods based on recombinant DNA.[1] A limitation of chemical modification is that the reagents do not possess a sufficient degree of specificity to produce pure materials in high yields.[1-3] Even reagents which are specific for particular functional groups of peptidyl side chains will usually produce heterogeneous mixtures since in a typical protein there are many occurrences of each amino acid type.[1-3] On the other hand, if a new functional group with unique reactivity can be introduced at a specific site in a protein, it should be possible to create a large variety of specifically modified versions of the protein.

We recently reported that the bulky, anionic trifunctional electrophile, trimesoyl tris(3,5-dibromosalicylate), TTDS, reacts preferentially with the ε-amino group of β-Lys-82 although there are 24 available amino groups per αβ dimer.[4]



TTDS

The major product from the reaction of TTDS with deoxyhemoglobin is the trimesyl bis(amide) from the ε-amino groups of Lys-82 of each β subunit, 3,5-dibromosalicyl trimesyl-((Lys-β-82)-(Lys-β-82))-hemoglobin (DBST-Hb). A schematic structure is shown below.



DBST-Hb

The presence of an unreacted ester group from the reagent in the specifically modified protein provides a unique reaction site which should permit the creation of a varied set of conjugates of the protein. As a demonstration of the possibilities of application of such a methodology, we have produced an illustrative group of conjugates.

Experimental Section

General. TTDS was prepared according to our previously reported procedure.[4] Biotin hydrazide, D-(+)-glucosamine hydrochloride, gly-gly-gly, lysine, and norepinephrine were purchased from the Sigma Chemical Co. HPLC-grade acetonitrile was obtained from Fisher Scientific. Buffers were made from water which was doubly distilled and deionized. Other reagents for preparation of buffers and developers for hemoglobin modifications and chromatography were all of analytical grade or better. Solutions of human hemoglobin were from Hemosol, Inc. Isolated yields reported were not optimized. Ion spray mass spectra were provided by the Analytical Service Group of the Biotechnology Research Institute, Montreal. Samples for analysis were prepared by lyophilizing solutions of the modified hemoglobins after ion-exchange chromatography. All samples were treated with 5% acetic acid for mass measurement.

Chromatography of Modified Hemoglobin Derivatives. Heme and globins were separated by reversed-phase HPLC using a 330-Å pore size C-4 Vydac column (250 × 4.6 mm, The Separations Group).[5] Developers contained 0.1% trifluoroacetic

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acid; gradients of acetonitrile were begun at 20% (v/v) and ran to 60%. The effluent was monitored at 220 nm. Separations of hemoglobins as intact tetramers were done by anion-exchange HPLC with a SynChropak AX300 column (250 × 4.6 mm for analytical and 250 × 10 mm for preparative) using developers containing 25 mM Tris and 25 mM Bis-Tris with pH gradients from 8.5 to 6.5, modified from the published procedure.[5] The effluent was monitored at 540 nm. After separation on the preparative column, the purity and identity of products were further confirmed by rechromatography on the C-4 and AX300 columns.

Polyacrylamide Gel Electrophoresis Analysis. This was performed on a MiniProtean II Ready Gel (12% polyacrylamide, 0.375 M Tris-HCl, pH 8.8) in the presence of 0.1% sodium dodecyl sulfate according to the procedure of Laemmli.[6] The hemoglobins from HPLC separation were prepared by heat denaturation in a buffer containing 65 mM Tris-HCl, pH 6.8, 2% SDS, 10% v/v glycerol, and 5% v/v 2-mercaptoethanol. Approximately 5–20 μg of protein was applied to each lane of the gel and processed at 200 V for 40 min. Protein bands on the gel were stained with Coomassie brilliant blue R-250.

Preparation of DBST-Hb. A solution of oxyhemoglobin (1.3 mM, in 4.5 mL 0.1 M MOPS, pH 7.2) contained in a 50-mL round-bottom flask was immersed in a water bath and connected to a rotary evaporator with the condenser replaced by a septum through which passed outlet and inlet tubes for gas flow. The oxyhemoglobin was converted to the deoxy form by passing a stream of humidified nitrogen over the solution in the rotating flask for 2 h at 37 °C. A solution of TTDS (1.0 mM, 7.0 mL, in 0.1 M MOPS, pH 7.2) was degassed and added under nitrogen to the rotating flask. The solution was maintained at 37 °C under flowing nitrogen for 2–3 h in the rotating flask to produce cross-linked deoxyhemoglobin. The protein solution was passed through a Sephadex G-50 column to remove unreacted reagent and other small molecules. The cross-linked deoxyhemoglobin was converted to the carbonmonoxy form by direct addition of carbon monoxide.

Products were analyzed by reversed-phase HPLC of separated globin chains and ion-exchange HPLC of intact tetramers. The solution of cross-linked hemoglobin thus prepared typically has a concentration of 0.3–0.35 mM. The structure of the material is the bis(amide) of trimesic acid formed from the 82-Lys ε-amino groups of the β subunits with the third acyl group of trimesic acid present as the ester of 3,5-dibromosalicylic acid.[4] The derivatized cross-linked hemoglobin dibromosalicyl bis(trimesylamide) is designated as DBST-Hb. For optimal use in the subsequent conjugation process, the solution was usually concentrated to 1.0–1.2 mM.

Acylation of Biotin Hydrazide by DBST-Hb. Biotin hydrazide (17 mg) was dissolved in 0.5 N HCl (0.5 mL) and 1.0 M MOPS (pH 7.2, 0.4 mL). The solution was brought to neutrality with 5% sodium bicarbonate (0.4 mL). Carbonmonoxy DBST-Hb (3.0 mL, 1.0–1.2 mM) was introduced, and the solution was incubated at 4 °C for 10 days. The solution was then passed through a Sephadex G-50 column to remove excess biotin hydrazide and other small molecules. The conjugation products were analyzed by a combination of reversed-phase HPLC of separated globin chains and ion-exchange HPLC of intact tetramers. The trimesyl–biotin–hemoglobin conjugate was separated on preparative ion-exchange HPLC and concentrated (yield: 32%).

Acylation of Glucosamine by DBST-Hb. D-(+)-Glucosamine hydrochloride (150 mg) was dissolved in 1.0 mL of 0.1 M MOPS (pH 7.2). Carbonmonoxy DBST-Hb (2.5 mL, 1.0–1.2 mM) was added, and the resulting solution was incubated at 4 °C for 7 days. Analysis and product isolation were performed by the method described above for the trimesyl–biotin–hemoglobin conjugate (yield: 38%).

Other Acylation Reactions of DBST-Hb. Lysine, gly-gly-gly, norepinephrine, and α-(1-nicotinamide)acetyl hydrazide were reacted with DBST-Hb, followed by chromatographic analysis and isolation of the resulting amides according to the procedures described for the biotin derivative. Formation of conjugates was readily observed in the appearance of new peaks in HPLC chromatograms. The isolated yields of conjugates were as follows: lysine (66%), gly-gly-gly (55%), norepinephrine (23%), α-(1-nicotinamide)acetyl hydrazide (49%).

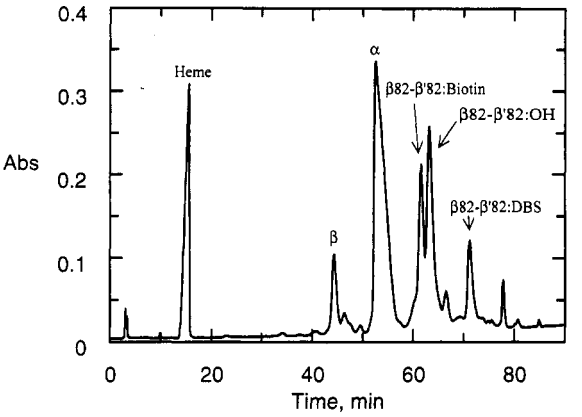

Figure 1. HPLC (C4 column) of separated subunits from partial reaction of DBST-Hb with biotin hydrazide.

Results and Discussion

Specifically Cross-Linking Hemoglobin with TTDS. As reported earlier, trimesoyl tris(3,5-dibromosalicylate) (TTDS) specifically attacks the hemoglobin DPG site, cross-linking Lys-82 on one β subunit to Lys-82' on the other to give the major product, DBST-Hb, in about 95% yield with no modification of the α subunits.[4] The remaining 5% of modified protein consists of the triply-linked material (trimesyl β-Val-1, β-Lys-82, and β'-Lys-82)[7] and that which is cross-linked between β-Val-1 and β'-Lys-82.[7] The latter are the major products in the reaction of deoxyHb with trimesoyl tris(methyl phosphate), an anionic acylating agent with smaller leaving groups.[5,7,8] These structures were identified by comparison of HPLC patterns. In the case of DBST-Hb, the third dibromosalicyl ester group remains unreacted, presumably as a result of steric and conformational restrictions which result after the two initial links are created.[4] The remaining ester in DBST-Hb undergoes a slow hydrolysis during cold aqueous storage ($t_{1/2} \approx 10$ days, 0 °C, pH 7.2) as monitored by HPLC.

Reaction of DBST-Hb with Nucleophiles. The reaction of biotin hydrazide with DBST-Hb gives a single modified protein which contains the acylated biotin hydrazide. Progress of the acylation reaction was followed by reversed-phase HPLC analysis under conditions which separate the globin chains of hemoglobin. The peak corresponding to the cross-linked β subunits decreases while a new peak for the biotin hydrazide derivative increases. The peak for the hydrolysis product of DBST-Hb also increases (Figure 1).

The formation of the trimesyl–Hb–biotin hydrazide product can also be seen by anion-exchange chromatography under conditions which separate intact hemoglobin tetramers. The conjugate appears as a peak in the chromatogram presented in Figure 2 along with the peak of the hydrolysis product of DBST-Hb.[4] Rechromatography of the material eluting in the peak assigned to the

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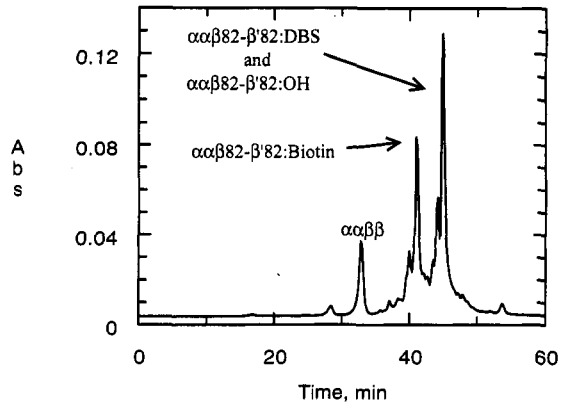


Figure 2. Anion-exchange chromatogram (AX-300) of intact tetramers formed from partial reaction of DBST-Hb with biotin hydrazide.

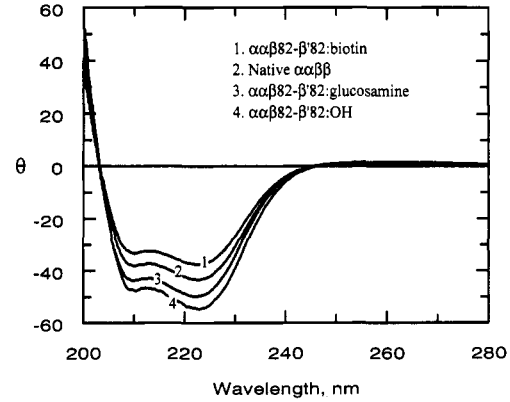


Figure 3. CD spectra of native and modified hemoglobins.

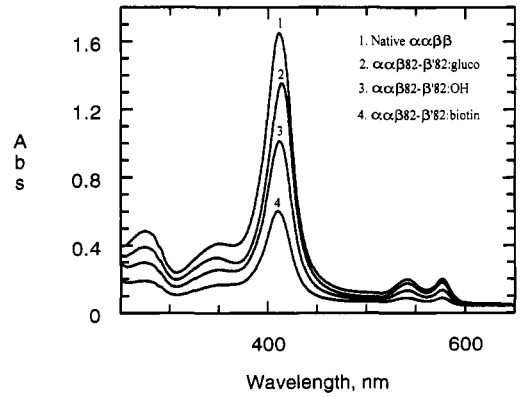


Figure 4. UV–vis spectra of native and modified hemoglobins.

acylated biotin hydrazide on the reversed-phase HPLC column coincided with the material which had been analyzed directly. SDS–PAGE of the biotin adduct showed a peak for the unmodified α chain as well as that of cross-linked β chains.

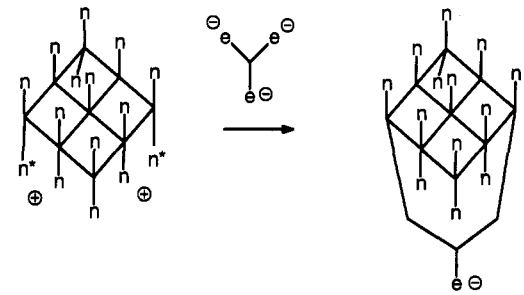
CD and UV–vis Spectroscopy. The CD and UV spectra of the biotin hydrazide adduct, ($\alpha\alpha\beta$82-β'82:biotin hydrazide), the hydrolyzed DBST-Hb ($\alpha\alpha\beta$82-β'82:OH) and native, unmodified Hb are virtually identical, suggesting that the tertiary structures of the cross-linked and conjugated Hb's are not significantly different from that of the unmodified native protein.[9]

Addition of Other Nucleophiles to DBST-Hb. By using the approach outlined above, additional conjugates of various types were readily prepared from DBST-Hb. Reactants included an amino acid (lysine), a tripeptide (gly-gly-gly), a vitamin analogue (nicotinamide hydrazide), a hormone (norepinephrine), and an amino sugar (D-(+)-glucosamine). The rates of the acylation reaction depend on the nucleophilicity and concentration of the added species. High inherent reactivity and high concentration of the nucleophile minimize the extent of the competing hydrolysis reaction of the hemoglobin adduct.

Characterization of the product from D-(+)-glucosamine was done by a combination of ion exchange chromatography and reversed phase HPLC. The molecular mass was determined by ion spray mass spectrometry.[10-12] The structural perturbation caused by the conjugation is also minimal as suggested by the CD[13] and UV–vis spectra[14] (Figures 3 and 4).

Ion Spray Mass Spectroscopy. As a general confirmation of the structural assignments several samples were analyzed. Parent mass peaks of the hydrolyzed ester of DBST-Hb ($\alpha\alpha\beta$82-β'82:OH) are 31 912.87 ± 1.59 for cross-linked β chains (calcd 31 911) and 15 128.44 ± 1.60 for unmodified α chains (calcd 15 126). Peaks given by the conjugate $\alpha\alpha\beta$82-β'82:biotin hydrazide are 32 152.29 ± 2.10 for the conjugated cross-linked β chains (calcd 32 153) and 15 127.85 ± 0.62 for unmodified α chains (calcd 15 126). The amide from the reaction of DBST-Hb with glucosamine ($\alpha\alpha\beta$82-β'82:glucosamine) gave a mass of 32 073.41 ± 1.27 for the conjugated cross-linked β chains (calcd 32 074) and 15 128.45 ± 1.12 for the unmodified α chains. This establishes that reactions occured irreversibly and that the HPLC chromatograms can serve as good indicators of product formation.

Scheme 1



Generalization

The reaction of hemoglobin with TTDS to form DBST-Hb takes advantage of the unique nucleophilic reactivity toward anionic electrophiles of the amino groups in the highly cationic site in hemoglobin which normally binds 2,3-diphosphoglycerate.[15] Acylation of two amino groups from each β subunit by TTDS generates an anionic electrophilic site on the protein itself. Thus, the reaction properties of the protein in the region change from being nucleophilic to electrophilic and the local charge from

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positive to negative. Since proteins do not normally contain ester linkages, the introduced ester presents a unique point of reactivity. By this process, acylation of any nucleophilic reagent becomes efficient. The overall concept is summarized in Scheme 1 where "e" denotes electrophilic sites (esters) and "n" denotes nucleophilic groups (amines).

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Abbreviations: Hb, human hemoglobin A; TTDS, trimesoyl tris(3,5-dibromosalicylate); DBST-Hb, 3,5-dibromosalicyl trimesyl-((Lys-β-82)-(Lys-β-82))-hemoglobin; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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