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THE EFFECT OF 4-ISOTHIOCYANATOBENZENE SULFONIC ACID ON THE OXYGEN AFFINITY OF HUMAN HEMOGLOBIN

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Summary

Human hemoglobin reacts with 4-Isothiocyanatobenzene sulfonic acid at the four amino groups of the N-terminal valines. The modified protein shows a decreased oxygen affinity over a wide pH range, a reduced alkaline Bohr effect, decreased co-operativity, and a reduced effect of inositol hexasulfate on the oxygen affinity.

Investigation of the change in functional properties in a protein such as hemoglobin as a result of chemical modification at determined sites can provide insight into the structure-function relationships and could lead to the design of chemical reagents that would alter the oxygen affinity in a predictable and therapeutically desirable way.

Chemical modifications of human hemoglobin at all four of the amino terminals with reagents such as cyanate (1) or (2) 2-methoxy-5-nitrotropone have characteristically led to a modified protein with increased oxygen affinity (1,2). Modification of only the β-chain termini with either: pyridoxal phosphate (3) or cyanate (1) was shown to produce a decrease in oxygen affinity. Reaction of only the α-amino termini with reagents such as cyanate (1) or 2,4-dinitrofluorobenzene (4) produced an increase in oxygen affinity. In this report we describe the results of the investigation of a reagent that reacts at all four amino terminals and produces a marked decrease in oxygen affinity. A general representation of the reaction is shown in Fig. 1.

Abbreviations: 4-ICBS, 4-isothiocyanatobenzene sulfonic acid

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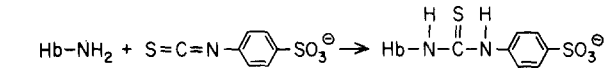


Fig. 1 - Reaction of 4-isothiocyanatobenzene sulfonic acid with hemoglobin ($\overline{HbNH_2}$).

Experimental

Materials: 4-Isothiocyanatobenzene sulfonic acid was synthesized according to the method of Dyson (5) as modified by Maddy (6). Inositol hexasulfate was purchased from Calbiochem (San Diego, CA). Fresh blood samples preserved in acid/citrate/dextrose were obtained from a blood bank. Human hemoglobin was prepared according to the method of Rossi Fanelli, Antonini and Caputo(7). The hemoglobin solutions were passed through a Sephadex G-25 column to remove 2,3-diphosphoglycerate before use.

Oxygen dissociation curves were determined spectrophotometrically (8).

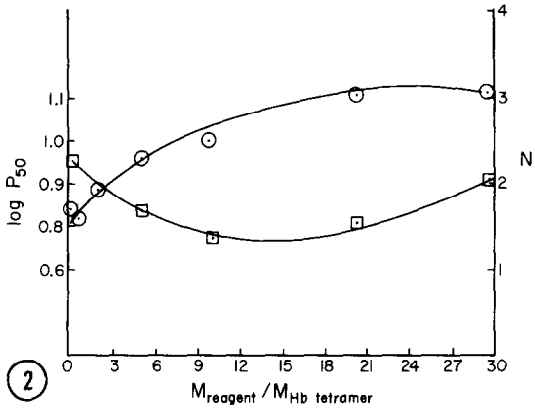
The 4-ICBS was added to a solution of oxyhemoglobin in either 0.1 M phosphate buffer, pH 7.4, or 0.5 M bis-tris buffer, pH 7.3 to give the desired ratio of reagent to hemoglobin tetramer, (usually 10/1). The solution was allowed to stand at room temperature for 5 h and then dialyzed against water for 24 h. In some experiments excess 4-ICBS was removed by passage through a Sephadex G-25 column.

The electrophoretic behavior of the reacted material was examined on cellulose acetate strips in 0.077 M tris/glycine, pH 9.1 plus 8 M urea for 1 h at 4°C with a current of 2 mA per strip.

In experiments designed to identify the modified amino groups globin A and globin A-4-ICBS which had been prepared according to the method of Rossi Fanelli (9) were aminoethylated according to the method of Cole (10) and then digested with trypsin (1/40, w/w) in 0.1 m NH_4HCO_3 solution at 37°C for 4 h. The tryptic peptides of aminoethylated globins were separated using High Pressure Liquid Chromatography (Perkin-Elmer "series 2") on a column of Lichrosorb RP 18 (10 μm) Merck with a gradient of acetonitrile in H_3PO_4, 0.1%. The elution profile was monitored at 210 nm. Amino acid analysis of isolated peptides was performed with an LKB 3201 Analyzer after hydrolysis in 6 M HCl for 24 h at 110°C. N-terminal analysis was carried out by the dansylation technique of Gray (11).

Results

The change in oxygen affinity as a function of concentration of 4-isothiocyanatobenzene sulfonic acid after 30 min reaction time and as a function of time at a reagent/hemoglobin concentration of 10/1 was determined. The effect on the oxygen affinity of increasing concentration of reagent is shown in Fig. 2. The oxygen affinity decreases with increasing concentration of the reagent while the n value at first decreases probably due to increasing heterogeneity and then returns to a value near 2. Experiments which monitored



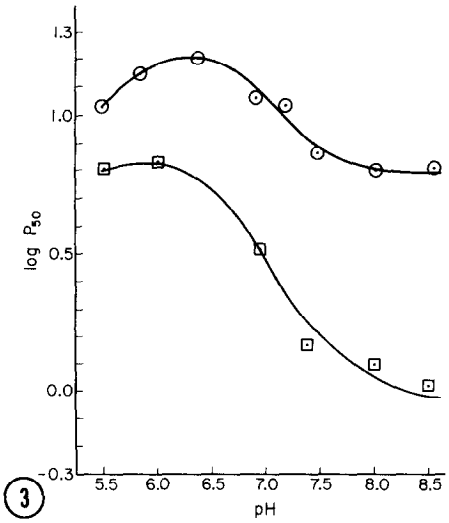


Fig. 2 - Oxygen affinity of hemoglobin (conc = 2.2 x 10^{-5} M) reacted for 30 min at 20°C with 4-ICBS in phosphate buffer, pH 7.4, with various concentrations of 4-ICBS, ⊙, log P_{50}; ⊡, n values.

Fig. 3 - Oxygen Bohr effect for HbA (conc 2.2 x 10^{-5} M) after reaction with 4-ICBS in 0.05 M bis-tris buffer, pH 7.3, ⊡, unreacted hemoglobin; ⊙, reacted hemoglobin, T = 20°C.

the change in oxygen affinity as a function of time indicated that the maximum effect had been reached after five h. Further, results of electrophoresis experiments indicated that the modified protein was homogeneous.

The effect of pH on the oxygen affinity of hemoglobin after treatment with 4-ICBS is shown in Fig. 3. The modified protein shows a decreased oxygen affinity over a wide pH range and a reduced alkaline Bohr effect.

Results of electrophoresis experiments indicated that both the α- and the β-chains of the modified globin had a greater anodic mobility that the α- and β-chains of untreated globin. These results indicate that both the α- and β-chains have reacted with 4-ICBS and are consistent with reaction with amino groups. The specificity of the reaction is demonstrated by the results of the following experiments: (i) Comparison of the elution profile of the tryptic peptides of the reacted globin with that of unreacted globin in Fig 4 shows that the modified peptides have a different chromatographic

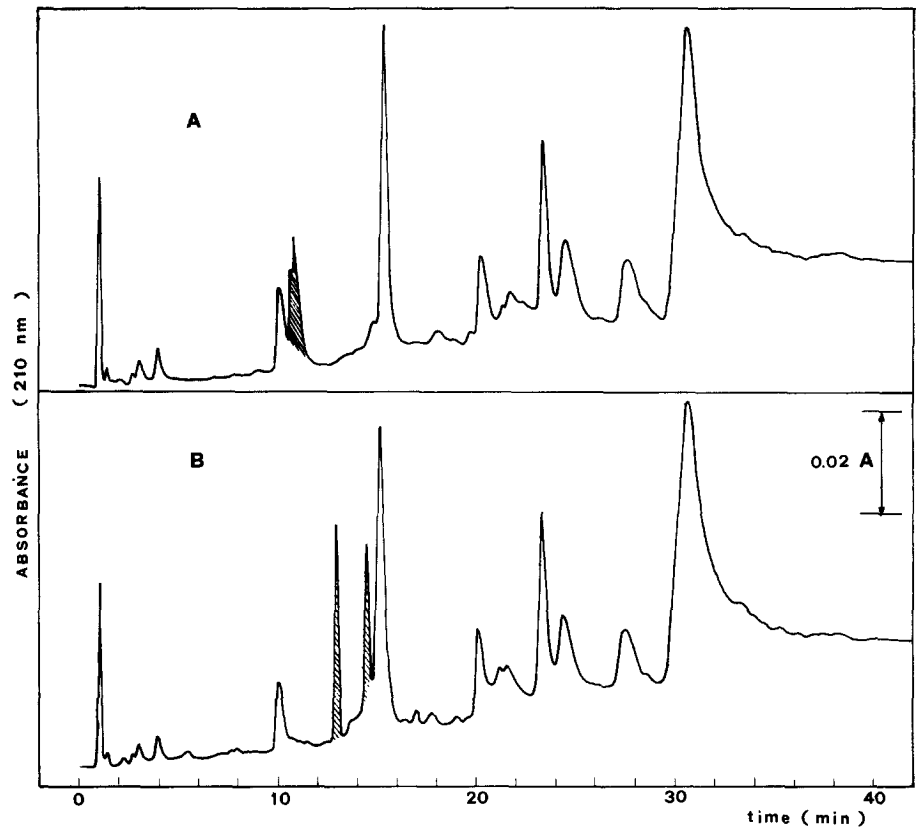


Fig. 4 - High pressure liquid chromatography elution profile of tryptic peptides of aminoethylated globins A, unreacted, and B, reacted with 4-ICBS on a Lichrosorb RP18 (10 μm) column with a gradient of acetonitrile in H_3PO_4, 0.1%, flow rate 3 ml/min; shaded areas, N-terminal peptides.

mobility and an increased absorbance at 210 nm. (ii) The amino acid analysis of the isolated peptides showed that the reacted peptides are the amino terminals of the α- and β-chains. (iii) Analysis of both reacted hemoglobin and globin by the dansylation technique indicated no free amino terminal groups.

The effect of inositol hexasulfate on the hemoglobin reacted with 4-ICBS can be seen in Table I. The effect of inositol hexasulfate is reduced but still significant. A similar result was reported by Nigen and Manning (12) in the effect of 2,3-diphosphoglycerate on hemoglobin carbamylated at the amino termini of the β-chains.

Table I

The Effect of Inositol Hexasulfate on the Oxygen Affinity of Hemoglobin Reacted with 4-Isothiocyanatobenzene Sulfonic Acid[a]

	log P_{50}	
Hemoglobin Derivative	No inositol hexasulfate	Inositol Hexasulfate, 8.8×10^{-5} M
HbA	0.29 (2.8)[b]	1.35 (2.2)[b]
HbA-4-ICBS	0.87 (1.7)[b]	1.13 (1.5)[b]

[a] Hb conc = 2.2×10^{-5} M (tetramer), 0.05 M Bis-Tris Buffer, pH 7.3 at 20°C

[b] n values in parentheses

Discussion

The decrease in oxygen affinity of human hemoglobin reacted with 4-isothiocyanatobenzene sulfonic acid is similar in magnitude to that observed by Benesch, Benesch, Renthal and Maeda for hemoglobin modified only at the amino termini of the β-chains by pyridoxal phosphate (13). These authors explained the decrease of oxygen affinity brought about by pyridoxal phosphate as due to "additional salt bridges formed with strategically placed positive residues such as lysine EF6β or histidine H21β." The distance between the nitrogen of one β-amino terminus and the 82 lysine of the opposite β chain is 11 A° (13). Approximation of the distance between the oxygens of the sulfonate moiety and the nitrogen of the β-amino terminal reacted with 4-ICBS using bond distances from model compounds (14) gives a value of 8.2 Å suggesting that some flexibility in the β-chains would be required to form the inter-chain electrostatic bond.

Reaction at the amino termini of the β-chains is consistent with the observed reduced effect on the oxygen affinity of the modified hemoglobin of inositol hexasulfate which is believed to bind in this region. Hemoglobin modified at the β-amino termini by pyridoxal phosphate has been reported to have an oxygen affinity unaffected by inositol hexaphosphate (15). The

observed decrease in the alkaline Bohr effect is consistent with reaction at the α-amino termini (16). The surprising result is that reaction at the amino termini of the alpha chains produces a modified hemoglobin with markedly reduced oxygen affinity since it has been proposed (17,18) that in deoxyhemoglobin the amino terminal residues of the α-chains form salt bridges with the C-terminal residues of the opposite α-chain. Rupture of this salt bridge would be expected to produce a destabilization of the deoxy form with subsequent increase in oxygen affinity unless a compensating salt bridge were formed. Fermi (19) has produced evidence that the distance between the terminal carboxyl and amino groups on opposite α chains is too great to permit a strong interaction.

The observed changes in the functional properties of hemoglobin reported herein afford an opportunity to study the importance of the steric and ionic charge relationships in the modifying reagent. Results of experiments with 3-isothiocyanatobenzene sulfonic acid, 4-isothiocyanatobenzoic acid and ethyl 4-isothiocyanatobenzoate will be reported elsewhere.

Acknowledgments:

This work is dedicated to Professor Alessandro Rossi Fanelli on the occasion of his seventy-fifth birthday.

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