Modification of Hemoglobin by Ninhydrin

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The Strecker degradation reaction was evaluated as a means of modifying hemoglobin in vitro, utilizing ninhydrin as a model compound. Ninhydrin led to modification of hemoglobin (when incubated with hemoglobin or red cells) at physiologic temperature and pH. Isoelectric focusing documented the formation of new hemoglobin bands, all with decreased (more negative) isoelectric points than hemoglobin A. Both alpha and beta chains were modified to an equal degree, although electrophoretic studies documented two modified species of alpha-chains and three modified species of beta-chains. Amino acid analysis of modified hemolysate following NaB₃H₄ reduction revealed peaks that coeluted with deaminated valine, deaminated lysine, and a product with the guanidino group of arginine. The oxygen affinity of hemoglobin increased following its incubation with increasing concentrations of ninhydrin. These studies suggest that ninhydrin is representative of a class of carbonyl compounds that could be utilized to specifically modify the structure and function of hemoglobin variants.

The observation that cyanate carbamylates amino groups of hemoglobin with resultant structural and functional changes has led to the study of a number of other agents that can covalently react with the amino groups of hemoglobin. All of the agents that have been described to date attach to amino functions by forming either reversible Schiff-base adducts or irreversible acetylated or carbamylated products. In considering other compounds that could modify hemoglobin in a specific way, we have studied a new class of reagents, exemplified by ninhydrin, that could act at physiologic temperatures to remove amino groups from the hemoglobin molecule.

MATERIALS AND METHODS

Preparation of Red Cells, Hemoglobin, and Ninhydrin Reaction

Hemoglobin was prepared from blood collected from normal volunteers. The red cells were separated from plasma by centrifugation at 800 g for 7 min at 4°C. The cells were washed 3 times with 5 volumes of isotonic phosphate-buffered saline (PBS), pH 7.4. Hemolysates were prepared by the addition of 2 volumes of distilled water to packed red cells. After 2-3 hr at 0°C, the red cell membranes were removed by centrifugation at 45,000 g for 4 hr in a Sorvall RC-5 Centrifuge. The hemoglobin supernatant was decanted from the membrane fraction, and its concentration was measured spectrophotometrically after conversion to cyanomethemoglobin using an extinction coefficient of ε = 1.1 × 10⁴ (heme basis) at 540 nm. Optical absorption was measured in a Zeiss PM-6 Spectrophotometer.

Hemolysates, stripped of organic phosphates, were prepared by dialysis in PBS in a stretched cellulose membrane against two changes of 1000 volumes of PBS, pH 7.4, for 24 hr at 4°C. Hemoglobin Aₙ was purified by cation exchange chromatography on Biorex 70 (BioRad, Richmond, Calif.), and purity was confirmed by isoelectric focusing as described below.

Red cells, hemolysates, or solutions of hemoglobin Aₙ were reacted with various concentrations of ninhydrin (Pierce, Rockford, Ill.) in PBS, pH 7.4, at 37°C for 2 hr unless stated otherwise.

Isoelectric Focusing

Hemoglobin was analyzed by isoelectric focusing on polyacrylamide gels with a pH gradient of 6-8. The modified hemoglobin species were quantified by scanning with an Ortec 4310 densitometer. Prior to scanning, the gels were stained with bromophenol blue and then dried.

Globin Chain Separation

Globin was prepared from control or reacted hemoglobin by precipitation in 2% HCl in acetone at ~20°C. Alpha and beta chains were separated by electrophoresis on strips of cellulose acetate in Tris-EDTA-borate buffer (133 mM, 5 mM, 15 mM, respectively), pH 8.9, containing 6 M urea and 0.07 M 2-mercaptoethanol. The strips were stained using an LKB 7000 fraction collector. The amount of radioactive tritium was determined in 5 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) in a Packard Scintillation counter. Standards from ninhydrin reacted with valine, α-t-Boc lysine, and α-t-Boc arginine (Bachem, Marina Del Rey, Calif.) were processed with the same procedure.

Amino Acid Analysis

Aliquots of control specimens or hemolysates that had been reacted with different concentrations of ninhydrin were diluted to 30 mg/ml and reacted for 10 min with a 200 μM excess of tritiated sodium borohydride (8 μCi/mmole) (Amersham, Arlington Heights, Ill.). Following extensive dialysis, 4 mg of the reduced borohydride globin was hydrolyzed in 6 N HCl at 110°C for 12 hr and analyzed on a Beckman 119C amino acid analyzer, using stream division to determine the tritium profile of the eluant. The stream division portion of the eluant was collected in 2-min fractions using an LKB7000 fraction collector. The amount of radioactive tritium was determined in 5 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) in a Packard Scintillation counter. Standards from ninhydrin reacted with valine, α-t-Boc lysine, and α-t-Boc arginine (Bachem, Marina Del Rey, Calif.) were processed with the same procedure.

Oxygen Equilibrium and Measurement of Methemoglobin

Hemoglobin oxygen saturation curves were obtained with a Hemo-Scan (American Instrument Co., Silver Spring, Md.). A 1.5-μl sample was deoxygenated by exposure to humidified nitrogen.
Fig. 1. Separation of hemoglobin reacted with ninhydrin by isoelectric focusing on polyacrylamide gel. Red cell suspensions (33%) and lysates (1.6 mM hemoglobin) were incubated with different concentrations of ninhydrin in PBS, pH 7.4, at 37°C for 2 hr.

for 15 min and then exposed to a continuously increasing oxygen pressure that was measured with a Clark oxygen electrode. The value of n and the P0.5 for the linear portions of the curves were calculated from the Hill equation by the method of least squares. Absorption spectra were recorded with a Beckman Acta C III spectrophotometer. Methemoglobin concentration was determined using the molar extinction coefficient, ε = 0.380 × 10^4 (heme basis), for pH 7.4 at 630 nm.

RESULTS

The reaction of ninhydrin was studied in vitro with hemoglobin in solution (1.6 mM) and with intact red cells (33% v/v). After 2 hr of incubation with 10 and 20 mM ninhydrin at 37°C, an array of new hemoglobin bands was present, all with more negative isoelectric points than hemoglobin A (Fig. 1). The amount of hemoglobin modified as well as the number of new more negatively charged bands on the gel increased with increasing concentrations of ninhydrin in the reaction mixture. The modification of hemoglobin in solution occurred to a greater extent at any given concentration of ninhydrin, than the modification of hemoglobin from intact cells (Fig. 2). After 2-hr incubation with 10 mM ninhydrin, 100% of the hemoglobin in solution was modified, but only 64% of the hemoglobin from red cells was altered as measured by densitometric gel scanning (Fig. 2).

In order to determine which of the hemoglobin subunits was modified, purified hemoglobin A was again incubated for 2 hr with 10 and 20 mM ninhydrin. Following heme removal, the globin chains were separated by electrophoresis (Fig. 3). Figure 4 depicts the densitometric scans of the electrophoretic patterns of the globin subunits of reacted and nonreacted sample. The integration of densitometric patterns for

Fig. 2. Percentage of total modified hemoglobin as assessed by isoelectric focusing after incubation of lysates (O—O) or red cells (●—●) in the presence of different concentrations of ninhydrin.

Fig. 3. Separation of globin subunits from samples of hemoglobin A0 incubated with ninhydrin. Hemoglobin A0 was reacted with 10 and 20 mM ninhydrin at 37°C in PBS, pH 7.4, for 90 min. The chains were separated on cellulose acetate with Tris-EDTA-borate buffer in 6 M urea at a pH of 8.55.

Fig. 4. Densitometric scans of globin chain separation on cellulose acetate gels. Peak 1 represents unreacted α-chains and peak 4, unreacted β-chains. Peaks 2 and 3 represent modified α-chains and peaks 5, 6 and 7 modified β-chains.
Fig. 5. A reaction scheme for the deamination of protein amino groups by ninhydrin and the incorporation of tritium label into the product. The N-terminal valines of globin (α or β chains – Prot – NH – R) are deaminated by ninhydrin, leaving a carbonyl group. The carbonyl group is subsequently reduced with tritiated borohydride. Tritium incorporation is measured following acid hydrolysis of the protein and amino acid analysis. The epsilon amino groups of intrachain lysines may be substituted in the scheme for the N-terminal valine.

Fig. 6. A profile of tritium radioactivity of hydrolyzed globin recovered from the amino acid analyzer. The profile of nonreacted globin is seen in the solid tracing. The three distinct new peaks of radioactivity in the ninhydrin-reacted globin are represented by the dashed lines, labeled 1, 2, 3. The peak time of elution of selected amino acids on the chromatogram are labeled above the graph.

The separated chains at 420 nm gave a ratio of optical density of α-chains to β-chains of 0.8 for the unreacted sample. This ratio remained constant in the reacted samples, indicating that each chain was modified by ninhydrin to the same extent. The modified species of α-chains (peaks 2 and 3) represent 34% and 56%, respectively, of the total chains following incubation with 10 and 20 mM ninhydrin concentrations, while the total modified species of β-chains (peaks 5, 6, and 7) represent 34% and 57% of the total chains.

The decrease in the isoelectric points of the modified hemoglobin components (Fig. 1) suggested that accessible amino groups were being removed or modified by ninhydrin. This hypothesis was investigated by performing amino acid analysis of the modified hemoglobin following reduction with tritiated sodium borohydride (NaB₃H₄) (Fig. 5). Tritium incorporated into ninhydrin-modified amino acids was detected in the eluant of the analyzer column for determination of radioactivity. As seen in Fig. 6, three distinct peaks of tritium were found in the ninhydrin-modified hemoglobin as compared with control. The new peaks were identified by determining the elution time of appropriate amino acids.

Fig. 7. Hill plots of oxygen equilibria of red cells after treatment with ninhydrin. The cells (33% suspension) were treated with ninhydrin for 90 min at 37°C in isotonic sodium phosphate buffer (120 mM) containing 5 mM glucose, pH 7.4, and washed extensively with the same buffer. The concentrations of ninhydrin used were 0 mM (x—x); 1 mM (●—●); 2 mM (Δ—Δ); 3 mM (○—○); and 5 mM (□—□). The oxygen equilibrium curves were formulated from a 33% red cell suspension in sodium phosphate buffer (120 mM) and glucose (5 mM), pH 7.4, at 37°C.
The percentage of right shift in the $P_{50}$ value of the hemoglobin oxygen dissociation curves as calculated from the equation:

$$\% \Delta P_{50} = \frac{P'_{50} - P''_{50}}{P'_{50}} \times 100$$

$P'_{50}$ and $P''_{50}$ are the partial pressures of oxygen at half saturation for the control and treated suspensions, respectively.

The reaction of ninhydrin with amino acids is frequently used as a colorimetric test for the quantification of amino acids. This method is based on the fact that ninhydrin promotes a deamination of α amino groups (Strecker degradation) with subsequent formation of colored products. Although most procedures are carried out on constituent amino acids at elevated temperature (usually 100°C), the reaction with proteins does proceed more slowly at lower temperatures.

The studies reported above show that hemoglobin in solution and within the red cell, reacts readily with ninhydrin at physiologic temperatures. The number of modified species resolved by isoelectric focusing indicate that there is not a specific site of reaction and that there is an even distribution of reacted species between α and β-chains. Amino acid analysis of globin that had been reacted with ninhydrin and reduced with sodium borohydride revealed that the sites of reaction were the ε-amino groups of lysine, the α-amino group of the N-terminal valine residues, and the guanidino group of arginine. The former two products are formed from the deamination of the ε-amino group of lysine and the α-amino group of N-terminal valine, respectively. The structure of the adduct of ninhydrin with the guanidino group of arginine is not proved but one has been proposed by Takahashi. It is important to point out that if there were other types of reaction between hemoglobin and ninhydrin that were not reduced by NaB₃H₄, these would not have been detected by our analysis.

Increasing the amount of modification of hemoglobin with ninhydrin decreases the oxygen affinity of the hemoglobin. In contrast, modification of the N-terminal residue of the α-chains with adducts has been shown to shift the hemoglobin conformational equilibrium toward the oxygenated state. However, since multiple sites of modification occur with ninhydrin, it is difficult to assign the effect on oxygen affinity to the modification of any particular amino group. As increasing the oxygen affinity of hemoglobin S results in a decreased rate of sickling, it would be of interest to determine the effect of ninhydrin on this particular hemoglobin.

The deamination reaction displayed by ninhydrin is representative of a series of carbonyl compounds (e.g., alloxan or isatin), which can deaminate amino groups by undergoing Strecker degradation. The work described above, using the model compound ninhydrin with red blood cells at physiologic temperature, provides impetus to incorporate this class of compounds into the overall strategy for the design of antisickling agents. It should be possible to identify new agents from this class of compounds that would be more specific for particular amino groups in much the same way as has been attempted for acylating agents.

DISCUSSION

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