BIOCHEMICAL PROPERTIES OF INTRAMOLECULARLY CROSS-LINKED HEMOGLOBIN

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Chemically modified hemoglobin (Hb) is nowadays regarded as the most probable basis on which to create an artificial oxygen carrier [2, 7]. Among the many chemically modified hemoglobins described in the literature, the one which has received the most study as a potential plasma expander is polyhemoglobin, obtained by the use of a bifunctional cross-linking agent — glutaraldehyde [4]. However, although polymerization of Hb can prolong its life in the blood stream [9], the optimal molecular weight of polyhemoglobin has not yet been established. The study of the polymer, during its circulation in the body, also has shown that an increase of molecular weight is not a factor which determines its retention in the blood stream [1], and it may also lead to an increase in its immunogenicity [5].

The biochemical properties of intramolecularly cross-linked Hb, and also its half-elimination time from the body, were studied in this investigation, for in this way the influence of structural changes of the protein molecule on circulation in the blood stream can also be established and an approach can be made to the problem of controlled modification with a view to obtaining an oxygen-carrying plasma expander.

EXPERIMENTAL METHOD

Intramolecular modification of Hb was carried out by the method described previously [8]. The Hb concentration in solutions and samples of blood plasma, and also their content of methemoglobin (met-Hb) were determined by an IL-282 CO-Oximeter (USA). Gel-penetrating chromatography of the modified Hb was carried out on columns packed with Sepharose CL 6B. Specimens of chemically modified Hb were subjected to isometric focusing in polyacrylamide gel within the range of pH 3.5-9.5, with a current of 25 A and power 25 W. The degree of

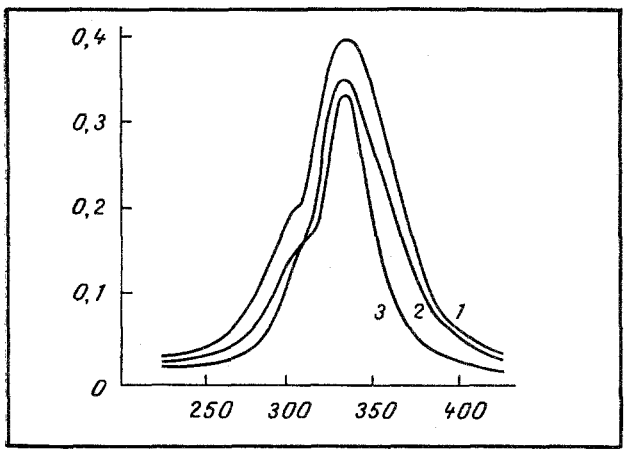

Fig. 1. Gel chromatography of chemically modified hemoglobin samples. Abscissa, volume of eluate (in ml); ordinate, optical density at 408 nm. 1, 2, 3) Serial Nos. of chemically modified hemoglobins. Carrier: Sepharose CL-6B; column 26 × 100 cm; rate of elution 25 ml/h; eluant 0.85% NaCl + 0.02% NaN_3; quantity of sample 50 mg; volume of sample 3 ml.

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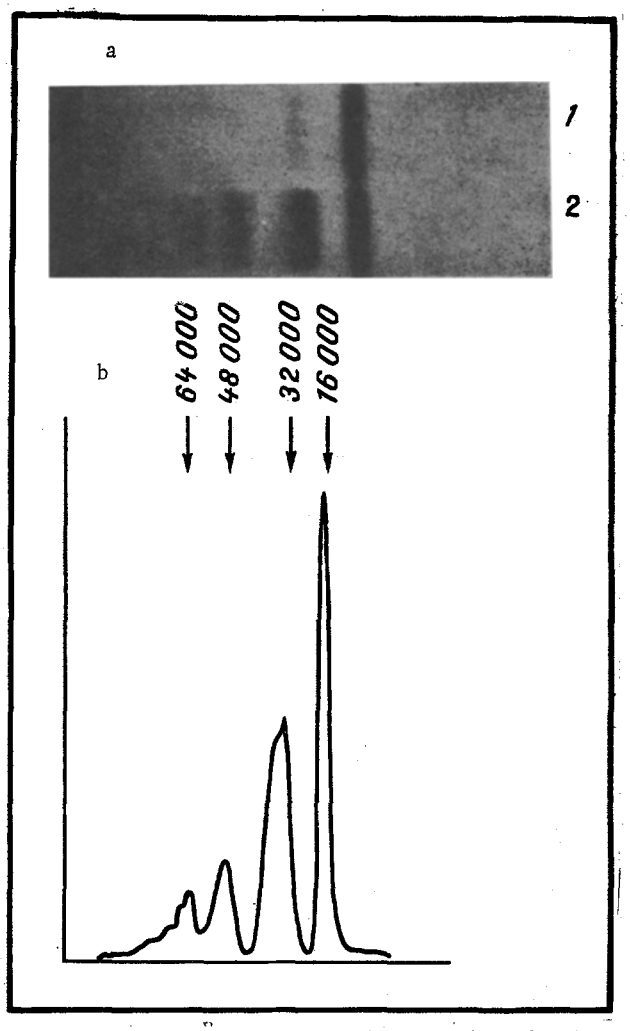

Fig. 2. Subunitary composition of intramolecularly cross-linked hemoglobin. a) Electrophoresis of native (1) and modified (2) hemoglobin; b) densitometric profile of intramolecularly cross-linked hemoglobin after electrophoresis. Ordinate, optical density at 603 nm; arrows indicate molecular weight of subunits.

cross-linking of the subunits was determined by Laemmli's method of SDS electrophoresis [3] followed by densitometry.

The oxygen dissociation curves were recorded by means of a "Hem-O-Scan" scanning analyzer (Aminco, USA) under physiological conditions (pCO_2 40 mm Hg, pH 7.4, 37°C, 0.15 M NaCl). The Na salt of pyridoxal-5-phosphate (PP) was titrated using a 1% solution of PP in 0.15 M NaCl, within the range of molecular ratios Hb:PP of 1:1 to 1:4.

Modified Hb was studied experimentally on 32 rats weighing 250-300 g. The animals were given an injection of about 6 ml of the test solution in a dose of 2.5 g/kg. Blood samples were taken 5 min and 3.6 and 18 h after injection. The data were subjected to statistical analysis on the HP 85 computer (Hewlett-Packard, USA).

EXPERIMENTAL RESULTS

Three series of chemically modified Hb, closely similar in composition, determined by methods of gel-filtration, SDS electrophoresis, and isoelectric focusing, were studied. The elution profiles of the synthesized samples given in Fig. 1 show that all were virtually free from components with mol. wt. of over 65 kilodaltons (kD). SDS electrophoresis revealed the subunitary composition of the synthesized compounds. Examination of the gels (Fig. 2) shows that the content of dimers and tetramers in the samples studied was 42-45% and 3-15% respectively, and the fraction of free subunits was 31-38%. Only subunits with mol. wt. of 16 kD were observed in the composition of the original Hb.

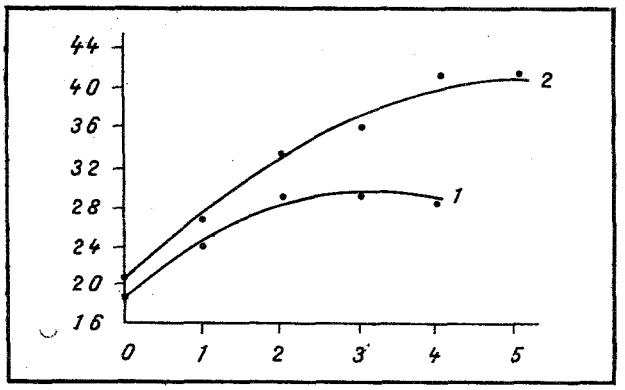

Fig. 3. Interaction of native (1) and intramolecularly cross-linked (2) hemoglobin with pyridoxal-5'-phosphate. Abscissa, value of p_{50} in mm Hg; ordinate, number of moles of pyridoxal-5'-phosphate per mole of Hb. Conditions: hemoglobin concentration 10%; pH 7.4; pCO_2 = 40 mm Hg; 37°C; 0.15 M NaCl.

The modification carried out, as the results of isoelectric focusing of the samples show, was virtually not reflected in the isoelectric properties of the chemically modified Hb, for which pI was 7.0-7.1 compared with 6.9-7.2 for unmodified proteins. The results show the presence of an intramolecular modification in the synthesized Hb derivatives.

To study the structural features of these compounds, their oxygen-transporting characteristics were determined and their interaction with allosteric effectors such as PP was investigated.

Measurements of the affinity of samples of intramolecularly cross-linked Hb or oxygen showed that the values of p_{50} were 18.5-20 mm Hg, virtually the same as the values of this parameter for the unmodified protein (p_{50} for Hb is 19.5 ± 1.5 mm Hg). Consequently, the modification which was done did not involve the functional group responsible for reversible addition of oxygen. Nevertheless, the results of titration of the test compounds with the aid of PP are evidence that the titration curves of intramolecularly cross-linked and native Hb are different (Fig. 3). In the first case saturation is achieved in the presence of only a twofold molar excess of the allosteric effector, the value of p_{50} being 27-30 mm Hg. For native Hb saturation arises with a molar ratio of Hb:PP = 1.4 and at a higher value of p_{50}. This is evidence of involvement of functional groups responsible for the regulatory effect of PP on reversible oxygenation of Hb in the process of protein modification by glutaraldehyde. These groups include the N-terminal groups of the β-chains β-82 Lys and β-143 His [6].

The study of the circulation time of the synthesized compounds in the blood stream of the rats showed that the half-elimination time for them was 4.5 ± 0.3 h, compared with 1.5-2.0 h for Hb. According to the results of SDS electrophoresis described above in comparison with the results of the biological tests, it can be concluded that a 50% content of undissociated dimers of subunits in modified Hb leads to a twofold increase in its life in vivo. Consequently, it can be postulated that controlled modification of Hb must ensure conditions for more complete dimer formation, which would probably lead to further lengthening of the circulation time.

An intramolecular modification of Hb by glutaraldehyde, with preservation of all the functions of the protein intact, was thus obtained for the first time and the influence of the subunitary composition of the molecule on its circulation time in the animal's blood stream also was established.

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EFFECT OF ACTIVATION OF THE ADENYLATE CYCLASE SYSTEM ON Na^+/H^+-EXCHANGE IN HUMAN PLATELETS

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Substances potentiating activity of the adenylate cyclase system in platelets inhibit the cell response to the action of various agonists [6]. An increase in the intracellular cAMP concentration depresses both the agonist-induced rise in the cytoplasmic Ca^{++} level [1, 2] and hydrolysis of phosphatidylinositol, leading to the formation of secondary messengers: inositol-1,4,5-triphosphate and diacylglycerol [9]. An essential role in the mechanism of platelet activation has been shown to be played by transmembrane Na^+/H^+-exchange, which is involved in elevation of the intracellular Ca^{++} level, the circulation of phosphatidylinositol, and aggregation and secretion [1, 4, 11, 12].

What is the effect of the adenylate cyclase system on Na^+/H^+-exchange in platelets? To solve this problem, we conducted an investigation on human platelets, in which the state of Na^+/H^+-exchange was assessed (by reference to changes in intracellular pH) in response to the action of aggregation inducers, phorbol ester (PMA, an activator of protein kinase C), and ionophores of monovalent and bivalent cations, before and after administration of carbacyclin (a stable analog of prostacycline), which raises the cAMP level in the cell [2].

EXPERIMENTAL METHOD

Venous blood from healthy donors, taken in a ratio of 6:1 with an anticoagulant of the following composition (in mM): sodium citrate 93, citric acid 7.7, glucose 140 (pH 6.5) — was used in the experiments. Platelet-enriched plasma (PEP) was obtained by centrifugation of the blood for 15 min at 150g. Platelets were isolated by centrifuging the mixture of PEP and anticoagulants (1:1) for 10 min at 350g and resuspended in buffer solution containing 138 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 0.2 U/ml of apyrase, 10 mM HEPES, and 0.2% bovine serum albumin, pH 6.5 (buffer A) up to a concentration of $(3\text{-}5)\cdot10^8$ cells/ml. The intracellular pH was measured by means of the fluorescent probe 2,7-bis-(carboxyethyl)-5,6-carboxyfluorescein (BCECF) by the method in [11]. For this purpose the pH-probe, in the form of the acetoxymethyl ester (BCECF/AM), was added to the platelet suspension up to a final concentration of 2.5-4 mM and incubated for 30 min at 37°C. The platelet suspension, diluted with anticoagulants (1:1), was recentrifuged for 10 min at 350g and resuspended in buffer A solution to a concentration of $(0.8\text{-}1.0)\cdot10^{10}$ cells/ml. Immediately before measurement, 10 µl of platelet suspension was added to 1 ml of buffer A solution (without albumin and apyrase, pH 7.4), or to 1 ml of a solution in which the NaCl was replaced by an equimolar concentration of choline chloride. Fluorescence was measured on a Hitachi 650-60 spectrofluorometer (Japan) at 22-24°C or 37°C. The wavelengths of excitation and emission

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