STRUCTURAL AND FUNCTIONAL HETEROGENEITY OF AN ARTIFICIAL OXYGEN CARRIER BASED ON HEMOGLOBIN

E. P. Vyazova, M. A. Azhigirova, L. V. Fetisova, A. L. Shuvalova, and A. I. Zeinalov

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Recent investigations have provided the solution to two basic problems connected with the creation of an artificial oxygen carrier based on extraerythrocytic hemoglobin, and determined by and concerned with the inefficiency of oxygen transport by the latter and its rapid elimination from the bloodstream through the kidneys [3]. By modifying hemoglobin with pyridoxal-5'-phosphate (PP) its gas-transporting characteristics were successfully brought near to those of human blood, and with the aid of subsequent polymerization with glutaraldehyde, the circulating time of the modified hemoglobin in the bloodstream could be increased to 24-48 h [1, 10]. The substance thus obtained, namely the pyridoxylated polymer of hemoglobin (PPHb), can be regarded as a potential substitute for erythrocytes of donated blood [10]. The main obstacle in the way of the practical use of PPHb, according to [8, 11], is its considerable heterogeneity as regards structural and functional properties, due to the statistical character of the chemical modification of the protein.

This paper describes preparative fractionation of PPHb by high-pressure liquid chromatography (HPLC), in order to study its fractional composition and to discover whether the optimal fraction can be selected on the basis of the following main criteria: molecular weight, functional properties, and immunochemical characteristics, which respectively determine the life span of the substance in the bloodstream, the efficiency of oxygen transport, and biocompatibility.

EXPERIMENTAL METHOD

PPHb was obtained by the method described by the writers previously [2]. PPHb was fractionated by HPLC on an Ultrapac TSK 545 DEAE column measuring 7.5 × 150 mm, in 0.05 Tris-HCl, pH 8.0, in a stepwise NaCl gradient from 0 to 1 M.

The molecular-weight composition of the fractions thus obtained was analyzed by HPLC on a Zorbax-250 9.7 × 250 mm column in 0.2 M phosphate buffer, pH 7.0. The column was calibrated with the aid of marker proteins (from "Bio-RAD") with mol. wt. of between 1300 and 670,000 D.

The oxygen dissociation curves were recorded on a Hem-o-Scan apparatus at pH 7.4, pCO_2 40 mm Hg, temperature 37°C, in 0.15 M NaCl solution.

The immunochemical properties of the fractions were investigated by confluent rocket immunoelectrophoresis in agarose gel against antiserum obtained by immunizing rabbits with PPHb in accordance with the following program: triple subcutaneous injection (at intervals of 2 weeks) of 1 ml of solution containing protein 5 mg/ml + 1 ml of Freund's complete adjuvant, a booster dose 4 weeks after the last injection, consisting of 1 ml of a solution of PPHb (10 mg/ml) + 1 ml of Freund's complete adjuvant. The serum content in the gel was 5%. Immunoelectrophoresis was carried out for 18 h under a voltage of 2 V/cm.

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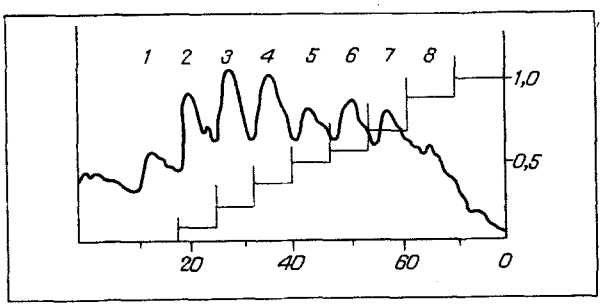

Fig. 1. Elution profile of PPHb on Ultrapac TSK 545 column. Abscissa, volume of eluate in ml; ordinate, optical density of eluate at 280 nm. Rate of elution 1 ml/min.

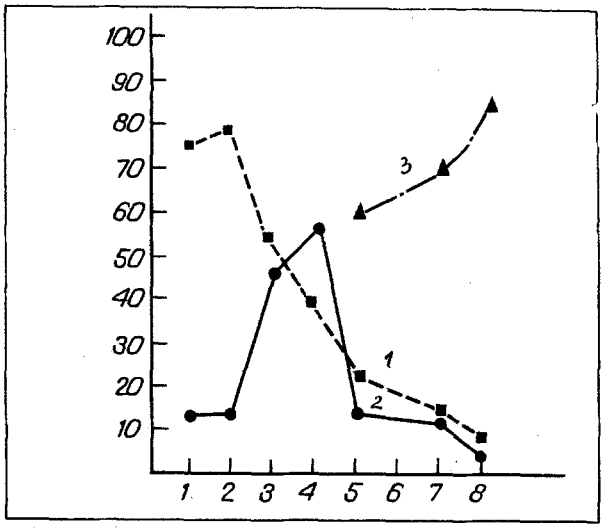

Fig. 2. Distribution of main components of PPHb by fractions: 1) component I (unpolymerized hemoglobin), 2) component II (120,000 D), 3) component III (over 350,000 D). Abscissa, Nos. of fractions; ordinate, content of components, in %.

EXPERIMENTAL RESULTS

The method of ion-exchange HPLC, used together with a stepwise NaCl gradient for preparative fractionation of PPHb yielded eight principal fractions. The elution profile and the gradient selected are illustrated in Fig. 1.

Analysis of the molecular-weight composition of these fractions revealed three principal components. The retention time of component I corresponded to that of native hemoglobin, whereas the retention time of components II and III responded to its modified derivative with mol. wt. of 120,000 and 350,000 D, respectively. As the data in Fig. 2 show, the ratio between these two components in the fractions chosen for analysis showed regular changes. For instance, the content of component I fell successively from its peak value of 76.9% in fraction 1 to 10.7% in fraction 8. The appearance of component II was observed as early as in the first fraction, of which it accounted for 14.3%, and reached a maximum (58.0%) in fraction 4; in subsequent fractions the content of this component diminished. Component III was found in only 5-8 fractions, in which its content rose from 60.2 to 86.5%. Thus the main fraction of the high-molecular-weight components was contained in the last four fractions.

Data obtained in the study of oxygen dissociation curves of the isolated fractions are given in Table 1. As the table shows, the functional characteristics of the isolated fractions differed with respect both to their P_{50} value, reflecting affinity for oxygen, and the degree of cooperativeness of the reversible oxygenation process, expressed by Hill's coefficient (n). The greatest value of P_{50} under these circumstances was observed for fractions 6 and 7. Incidentally, these values were rather lower than those

TABLE 1. Functional Characteristics of PPHb Fractions

Character-istics	Nos. of fractions							
	1	2	3	4	5	6	7	8
P_{50}, mm Hg	21,0	23,0	21,1	23,9	20,5	27,6	26,3	17,6
n	1,89	1,65	1,74	1,73	1,56	1,84	1,85	1,53

Legend. For unfractionated PPHb: P_{50} = 30 mm Hg, n = 2.16.

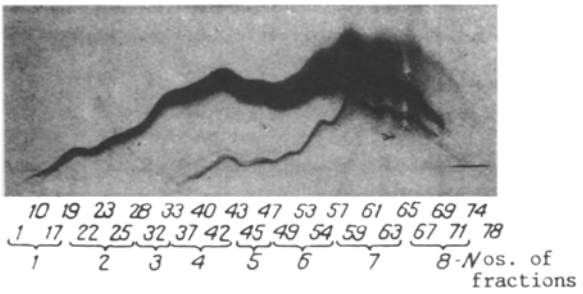

Fig. 3. Confluent rocket immunoelectrophoresis of PPHb fractions. To each well 10 μl of fractions with protein content of 0.1 to 0.3 mg/ml is added; agarose contains 10% rabbit antiserum to PPHb.

for the original polymer. This reduction in P_{50} may be connected with a disturbance of intermolecular interactions, taking place in the original PPHb, in the course of fractionation.

The considerable heterogeneity of PPHb, exhibited with respect to its structural and functional properties, suggested that these fractions make different contributions to the increase in antigenicity of hemoglobin as a result of its polymerization, observed by a number of investigators [4, 9]. This hypothesis is confirmed by the results of immunochemical investigations shown in Fig. 3. It will be clear from Fig. 3 that fractions 6-8, with new antigenic determinants responsible for the presence of additional precipitation profiles also can be observed to appear, possess the greatest antigenicity. These results correlate with those obtained during biochemical investigations of polymerized hemoglobin [7], and they are evidence that its heterogeneity is not confined purely to molecular weight, but the product obtained by polymerization of hemoglobin is also chemically heterogeneous.

Investigation of the fractional composition of PPHb by the three different methods demonstrated its heterogeneity with respect to structural characteristics, and also to functional and antigenic properties. The difference between the functional properties of the isolated fractions is associated with the degree of modification of hemoglobin by PP, which can vary from 2 to 6 moles per mole of protein [6]. Structural heterogeneity of PPHb is determined by nonspecificity of the polymerization reaction, with the use of glutaraldehyde, leading to attainment of a heterogeneous population of cross-linked monomers and polymers. Structural differences between fractions of PPHb, determined by this polydispersed state, are reflected in antigenic properties. The results are evidence that the increase in mol. wt. of PPHb within the range of 100,000-200,000 D, taking place in fractions 1-4, is not significantly reflected in their immunochemical properties. Meanwhile, for fractions containing a component with mol. wt. of over 350,000 D increased antigenicity and the appearance of new antigenic determinants are observed. This is in agreement to some extent with data in the literature [5], according to which the presence of high-molecular-weight components in the composition of PPHb is one factor negatively affecting its biocompatibility.

On the basis of these results we can identify fractions of PPHb that are optimal with respect to the three criteria studied: molecular weight, functional activity, and antigenic activity. Fractions of this kind are 3 and 4, which do not contain high-molecular-weight components, which have P_{50} of about 23 mm Hg, and possess no antigenic activity that differs qualitatively from that of the original hemoglobin, and which also are free from significant amounts of poorly circulating nonpolymerized

hemoglobin. The value of P_{50} of the selected fractions, although not the peak value (since it is maximal in fractions 6 and 7, which contain a high-molecular-weight component), nevertheless is sufficient to ensure effective oxygen transport in vivo [10].

As regards biocompatibility, however, and in particular, considering the criteria of antigenic activity and toxicity, these fractions are preferable to the original PPHb.

Thus the observed heterogeneity of PPYHb is not an unsurmountable drawback, as has been stated in [8], for the choice of an appropriate method of fractionation can result in the isolation of an optimal fraction, with a high degree of homogeneity of its structural and functional properties.

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