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Crystalline Leghemoglobin

VI. The Comparison of the Two Main Components by Tryptic Peptide Pattern Analysis

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The two main components of leghemoglobin have been subjected to tryptic hydrolysis. The resultant peptides have been separated in two dimensions by paper electrophoresis and paper chromatography. Comparison of the peptide patterns of the two components shows many identical peptides, the differences, however, being rather great including several peptides. It is concluded from the results that the two components of Lhb are formed by synthesis by systems that differ genetically.

The concept of the two main components of leghemoglobin (Lhb) being individual proteins is based on recent studies on molecular weight [1], amino acid content [2] and N-terminal amino acids [3]. There are two ways by which these individual components might be formed. One way might be by synthesis *via* individual systems that differ genetically. The other might be a system in which one of the components acts as a precursor for the other. The former possibility has support in the observation that those amino acids, the content of which has been found to differ between the two components, are identical with those amino acids which are known to be interchangeable in specific proteins of different origin, *i.e.* which are synthesized by systems differing genetically. The other possibility, *i.e.* one of the components being a precursor for the other, has particular support in the fact that the two components have different N-terminal amino acids and a slight difference in molecular weight.

In order to elucidate the structural differences between the two components, and to reach a final conclusion as to whether one of the components acts as a precursor for the other or if the two components are synthesized by two

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systems under different genetic control, it was decided to study the pattern of their tryptic peptides. The analyses were performed by combined paper electrophoresis and paper chromatography, a technique introduced by Ingram [4] as "the finger printing technique" for detecting small differences in amino acid composition or sequences in proteins, differences which are of importance in the composition of homogeneous proteins from different animal and plant species and those which result from a single gene mutation.

MATERIALS AND METHODS

Leghemoglobin. The two main components of Lhb used in this study were prepared as described previously [5,6]. The electrophoretic homogeneity of the components was checked.

Apoleghemoglobin. The apoproteins of the two components were prepared from the electrophoretically homogeneous components by precipitating in HCl-acetone in the cold as described previously [2].

Trypsin. The trypsin used for the digestions was the crystallized protein salt obtained from Worthington (cryst. Trypsin) and was further purified by electrophoresis in order to remove the chymotrypsin present in the preparation. A typical run was made in the Spinco model E electrophoresis apparatus as follows: A 1.3 % solution of the trypsin was run in the 2 ml cell in a phosphate buffer pH 6.27 and μ 0.1. The preparation showed two components, a main component and a smaller amount of a very slow component. The main component moved to the cathode with a rather high mobility and was found to contain all of the tryptic activity, whereas the smaller component was found to contain the chymotryptic activity. After about 5 h, the separation of the two components had proceeded so far that enough electrophoretically pure trypsin could be isolated. This was done after first adjusting the positions of the two components so that the trypsin-containing component could be removed with a hypodermic needle introduced into the electrophoresis cell. This trypsin preparation showed only slight traces of chymotryptic acitivity.

Assay of chymotrypsin. The chymotrypsin content of the different trypsin preparations was determined by using N-benzoyl-DL-phenylalanine-β-naphthyl ester as a substrate as described by Ravin *el al.* [7].

Assay of trypsin. The trypsin activity of the samples was determined according to Kunitz [8]. 0.5 % casein solutions were hydrolyzed at 35°C for 20 min, the excess casein precipitated with trichloroacetic acid, and the concentration of the split products in the supernatant solutions determined from the optical density at 280 mμ. The amount of active trypsin was determined from the standard curve of Kunitz.

Digestions. The tryptic digestions were performed essentially as described by Ingram [9]: 6.5 ml samples of 0.2 % Lhb solutions were first denaturated by heating in a salt free solution at pH 9 for 5 min at 90°C. The digestions were carried out with continuous stirring at 39°C and pH 9 in an atmosphere of nitrogen. The enzyme to Lhb ratio was 0.01, with the trypsin concentration calculated as active trypsin. The pH was maintained constant by adding 0.2 N NaOH from an Agla micrometer buret. The digestion was continued for 140 min. After digestion, the pH of the solution was brought to pH 4.0 with 4 N acetic acid, after which the solution was lyophilized. These peptide preparations, particularly the hydrolyzate of the faster component, showed a tendency to gelatinize on dissolving in water. The gelatinization tendency was decreased by redissolving the preparations in water, removing the gelatinized material by centrifugation and relyophilizing the supernatant solution.

High-voltage paper electrophoresis. The apparatus consisted of a brass plate coated with a thin layer of polyethylene, and cooled by a system consisting of copper tubing under the plate connected to the tap water [10]. The plate was large enough for a whole sheet of Whatman 3 MM to be used. The electrophoretic separation was carried out in 0.03 M pyridine-acetic acid buffer of pH 4.0 on large sheets of Whatman 3 MM, and at a potential of 27 V/cm. In order to stabilize the temperature of the paper, the current and cooling water were turned on 20 min before the run was started.

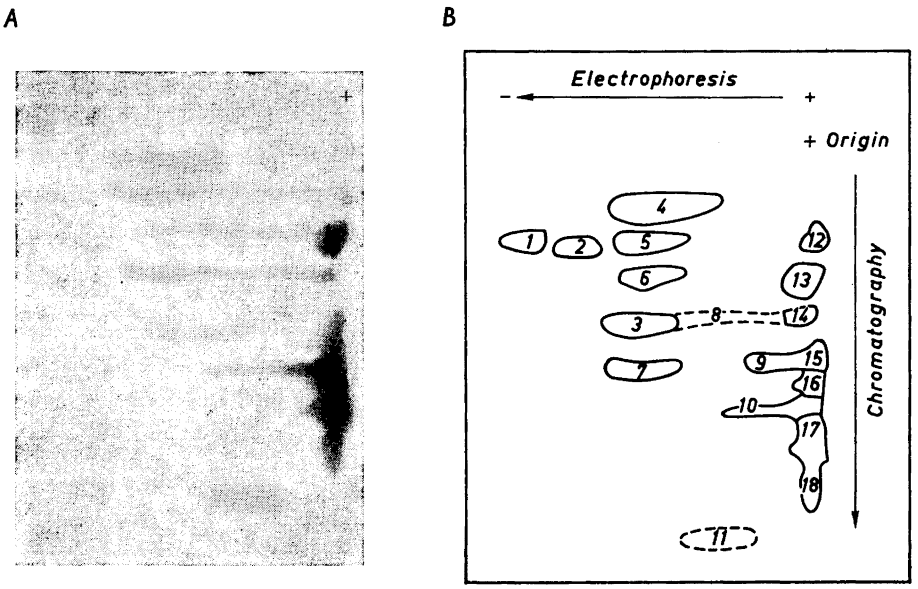

Fig. 1. Two-dimensional separation of tryptic peptides of the faster component of Lhb (A) and its schematic presentation (B). The first dimension is electrophoresis at pH 4.0. The second dimension is chromatography in butanol-acetic acid-water.

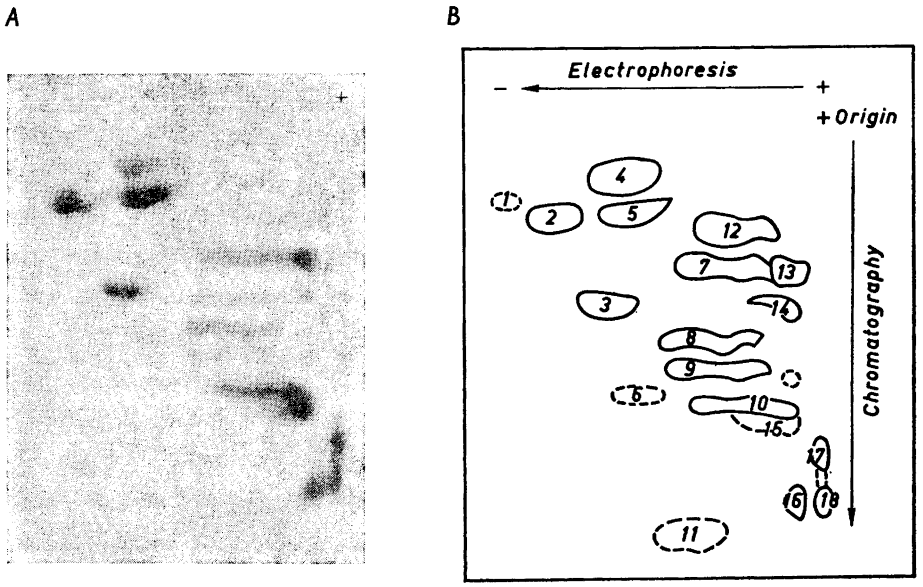

Fig. 2. Two-dimensional separation of tryptic peptides of the slower component of Lbh (A) and its schematic presentation (B). The two dimensions are the same as in Fig. 1.

Peptide pattern analysis. The tryptic hydrolyzates were analyzed by the combination of electrophoresis and chromatography on paper as specified by Ingram [9]. After the temperature of the paper on the plate was stabilized, the sample was put on. The area around the place of application was dried with "Whatman filter paper accelerators" of 1.95 cm diameter [10]. 2—2.5 mg of the tryptic hydrolyzates in 20 μl were put on the dried areas. The electrophoresis was continued for 120 min. The paper was dried, and subjected to descending chromatography in butanol-acetic acid-water (4:1:5 volume ratios) for 6 to 7 h at 37°C in a thermostated room. The spots on the chromatograms were developed by dipping the papers in a 0.4 % ninhydrin solution in acetone containing 2 % pyridine and left at 37°C for color development.

RESULTS

Figs 1 and 2 show tryptic peptide pattern of the faster and slower components and their schematic representation. The polarity of the electrical field is marked on each pattern, and the point of application of the sample is designated by a cross (+).

The fingerprints contain alltogether some 18 peptides. As judged by their ninhydrin colors, the majority are present in good yield. There are slightly more peptides than expected from the amino acid compositions of the two components. Trypsin is well known to split specifically those bonds in the polypeptide chains which are formed by the carboxyl groups of lysine and arginine. There are 13 lysine residues and 1 arginine residue in the fast component, and 13 lysine and 2 arginine residues in the slow component. According to these figures, 14—15 peptides are expected to be formed from the faster component and 15—16 peptides from the slower one after complete trypsin digestion (depending on whether or not there is a lysine or arginine residue as a carboxyl end-group). However, as the amino acid analyses do not account for more than 92.7 % [2] of the apoprotein for the faster component and 95.7 % [2] for slower one, the possible presence of constituents other than amino acids must also be kept in mind.

Peptides numbered 1, 2, 3, 4, 5, and 11 in the digests of both the two components occupy the same relative positions and are therefore considered to have the same chemical structure. Peptides numbered 6 and 7 in the digest of the fast component cannot be found in the digest of the slow component. Peptide number 6 of the fast component might correspond to peptide 7 of the slow component. However, the differences in the peptides of the two components are too great to allow any further conclusions to be drawn on possible changes of the positions of the amino acids in the peptides. A general feature is that the neutral peptides of the fast component (Nos. 12—18) occupy a slightly changed position in the digest of the slow component. This is in part due to a slight increase in the positive electrical charges in the corresponding peptides of the slower component. This finding agrees well with the known electrophoretic behavior of the two components.

The spots numbered 2 in both of the components and that numbered 7 in the fast component were found to turn yellow when kept at room temperature long enough so that the red color of the ninhydrin practically disappeared. This behavior seemed to be reproducible and was therefore assumed to be

specific for the peptides. Perhaps the proline in these peptides is so situated that it is accessible for color formation with ninhydrin (perhaps as an amino endgroup).

DISCUSSION

When the results presented in this paper are examined, the possibility of one of the components being a precursor for the other must be excluded. Instead, it is assumed that the two components are synthesized by systems which differ genetically.

Recent studies on normal and abnormal hemoglobins [11–13] have given valuable insight into the action of a gene and in particular into the firm control exerted by a gene. The chemical structure of a gene is thought to determine the amino acid sequence of the corresponding peptide chain. Any alternation of the gene, *e.g.* a mutation, would result in a chemical change in the peptide chain.

A chromosomal difference has been observed between the cells of the root nodules and those of the root tip. The root-tip cells had the usual diploid number of 14, whereas the root nodules had the tetraploid number of 28 [14]. The doubling of the chromosome number seemed to be associated with the bacterial invasion of the plant cell. It is probably not related to nitrogen fixation. Since nodules formed by inefficient strains also had a double chromosome number as compared with that of the root tip [15], it is difficult to decide the extent to which the synthesis of the two Lhb components is related to it, particularly as a positive correlation exists between the Lhb content in the nodules and the nitrogen fixation [16,17].

The nodule bacteria may, however, cause such changes in the plant cells that the components with chemical difference might be synthesized. It seems less likely that one of the components should be synthesized by the plant cell and the other by the bacterial cell. Recent findings by Bergersen and Briggs [18] revealed a highly organized structure in the soybean root nodules. Electron photomicrographs of mature, nitrogen-fixing nodules showed a system consisting of groups of four to six bacteroids enclosed in well-defined double membranes. It seems unlikely that equal concentration of the two components should always be synthesized on both sides of the double membrane, one component inside the menbrane by the bacteria and the other outside the membrane by the plant cell.

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