Crystalline Leghemoglobin

V. The N-Terminal Amino Acids of the Two Main Components

NILS ELLFOLK* and KLAS LEVIN

Medicinska Nobelinstitutet, Biokemiska Avdelningen, Karolinska Institutet, Stockholm, Sweden

Recent studies have shown that the two main components of leghemoglobin (Lhb) differ sligthly in respect to their molecular weights [1] and amino acid contents [2]. This indicates that they are individual proteins. Long-run electrophoresis at different pH values [3] showed them to be homogeneous. In order to get additional information on the homogeneity of the two components, it was decided to study their N-terminal amino acids using Sanger's 1-fluoro-2,4-dinitrobenzene (FDNB) method.

Experimental. The two main components of Lhb were prepared as described previously [3], and their homogeneity was electrophoretically checked. Different conditions of coupling with FDNB were tested, including the ethanol-bicarbonate and the ethanol-trimethyl-amine media, and it was decided to perform the coupling by stirring 0.2 μmoles of the dialyzed protein component in 3 ml of 0.1 M KCl solution with 100 μl FDNB at 40°C [4]. The pH of the solution was kept constant at 8.9 by successively adding 0.1 N NaOH from an Agla micrometer burett. The reaction was practically complete after 40 min, but was continued for still another 20 min during which the alkali consumption showed a linear course. The reaction mixture was then extracted with peroxide-free ether, after which the DNP-protein was precipitated by bringing the reaction mixture to pH 1.5 by adding 1 N HCl. A difference between the DNP-derivatives of the two components was observed in their ability to redissolve from the acid precipitate when the pH was increased. The DNP-derivate of the electrophoretically faster component went into solution easier than the derivative of the slower one. After the acid precipitation, the precipitates were washed with anhydrous ethanol and acetone, and stored in an evacuated desiccator over phosphorus pentoxide.

The DNP-proteins were hydrolyzed under varying conditions to meet the requirements for the detection of the more labile DNP-amino acids as well as those which are relatively stable. The hydrolyses were performed with HCl in the common way, as well as with a mixture of formic and perchloric acids in presence of acetic acid anhydride (1.0 : 015 : 055 by volume) according to Hanes *et al.*[5] Tubes with 0.2—0.3 ml of the acid and 0.2 μmoles of the DNP-protein were kept for varying times at 105° and 100°C for the HCl and formic-perchloric acid mixtures, respectively. The hydrolyzates were diluted with water and extracted in glass-stoppered test tubes with ether when the hydrolysis had been performed with HCl, and with benzene-*tert*-amyl alcohol (9:1 by volume) when it had been performed according to Hanes *et al.*[5] Each extract was washed with water to remove any acid-soluble material such as ε-DNP-lysine. The material which was soluble in ether or benzene-*tert*-amyl alcohol was transferred to Whatman No. 1 paper for two-dimensional chromatography according to Levy, as modified by Wallenfels and Arens [6]. The aqueous phase was examined by electrophoresis in 1 N NH_3, and by chromatography in *tert*-amyl alcohol — pH 6 phthalate [4].

For quantitative determination, the spots were eluted with 1 % sodium bicarbonate solution, and the amount of DNP-amino acid in the eluate determined spectrophotometrically. Readings of the optical densities were taken at 360 mμ, and the amounts of the amino acid derivatives were calculated using the extinction coefficient at 360 mμ of Wallenfels and Arens [6].

Results. In qualitative experiments, glycine was identified as the N-terminal amino acid for the electrophoretically faster component of Lhb, whereas valine was found to be the N-terminal group for the slower one. No other ether-soluble α-DNP-amino-acids could be identified on the chromatograms of the hydrolyzates of the DPN-derivatives of the two components. No positive evidence for the occurrence of histidine or arginine in the N-terminal position in the two components could be obtained.

Because of the well-known lability of DNP-glycine to hydrolysis, the DNP-derivative of the faster component was subjected to hydrolysis under various conditions. Short-time hydrolysis with formic and perchloric acids [5] was not found to be as quantitative with Lhb as was expected from the studies on the glycine peptides [5]. The hydrolysis is not complete under the conditions recommended (2 h at 100°C)

* Permanent address: Research Laboratories of the State Alcohol Monopoly, Helsinki, Finland.

since the extracts of hydrolyzates showed on chromatography a yellow streak which hardly moved in the phosphate-buffer system of Levy [4]. Chromatography of the aqueous phase in *tert*-amyl alcohol revealed the presence of ninhydrin-positive yellow spots which were obviously DNP-peptides of varying size. In addition, the yield of ε-DNP-lysine was very low. The incomplete hydrolysis was, therefore, one reason for the rather poor yield of glycine, which corresponded to 21 % of the total glycine present as an end group. The hydrolysis of the DNP-derivative of the faster component with 6 and 12 N HCl gave such low yields of DNP-glycine that it could not be measured. In comparison, the hydrolyzates of the DNP-derivative of the slower component showed on chromatography no derivatives which could be assumed to be DNP-peptides. The recovery of DNP-valine in the slower component was found to be 60 % after a 24 h hydrolysis at 105°C in 6 N HCl. According to Porter and Sanger [7], DNP-valine was 64 % unchanged in a 24 h hydrolysis in 5.7 N HCl. Using this value as a correction for the recovery of DNP-valine, a yield of 0.93 moles of valine was obtained for one mole of protein.

Discussion. The number of free α-amino groups gives the number of the open peptide chains present in the protein molecule. From the above findings it is evident that the electrophoretically-faster component of Lhb consists of one peptide with N-terminal glycine, whereas the slower one consists of one chain terminating in valine. These findings agree well with recent conclusions drawn from studies on the amino acid contents of the two components. The total lack of sulfur-containing amino acids, and accordingly the absence of S—S bonds that could form bridges between different peptide chains, led to the conclusion that the two components consist of one peptide chain only. Comparative studies on the N-terminal amino acids of normal and fetal human hemoglobin, as well as on the red cell pigments of various animal species, have shown that the number of peptide chains varies from two to six per molecule. It also seems that all hemoglobins contain at least one invariant chain terminating in valine [7,8]. In the case of myoglobins, which have a single peptide chain, the data are limited. Horse myoglobin has been found to terminate in glycine [7,9] in all the fractions (Mb I, Mb II and Mb III), while whale myoglobin [10] terminates in valine. The interchange of glycine and valine in the N-terminal position as found above in the two Lhb components gives additional support to the assumption recently put forward [2] that the synthesis of the two components is controlled by systems which are genetically different.

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Derivatives of 4-Amino-2-hydroxybenzenesulfonic Acid

K. A. JENSEN and O. BUCHARDT

Chemical Laboratory of the University of Copenhagen, Copenhagen, Denmark

In connexion with researches [1-6] on derivatives of 4-amino-2-hydroxybenzoic acid (PAS) we wished to investigate the tuberculostatic activity of the analogous 4-amino-2-hydroxybenzenesulfonic acid and its derivatives. According to Lespagnol, Sevin and Beerens [7] the free acid is without tuberculostatic effect, but Youmans (*cf.* Doub *et al.*[8]) found both the acid and its amide to be slightly active. As the tuberculostatic effect of PAS is — at any rate in part — connected with an antagonism to *p*-aminobenzoic acid [1,9,10], it seemed not improbable that introduction of heterocyclic substituents into the sulfonamide group of 4-amino-2-hydroxybenzenesulfonamide might enhance the bacteriostatic activity of this compound very significantly just as it does with sulfanilamide. Our attempts to