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Note

Purification of the isolated β-chain of adult human haemoglobin from its post-translational modification

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In the haemolysate of adult human haemoglobin (Hb), some minor components are present, which probably originate from post-translational non-enzymatic glycation of β-chain NH_2 terminal groups [1] and are collectively termed HbA_{1a-d}, the α-chain, in contrast, probably owing to steric hindrance, undergoes minor terminal modifications. Thus, whenever the assessment of the functional properties of HbA is the aim of an experiment, cation-exchange chromatography of the whole haemolysate becomes necessary in order to obtain the pure HbA_0 component In fact, the percentages of HbA_1 components are variable and their properties are substantially different from those observed for HbA_0 [2]

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Moreover, according to the above-mentioned considerations, a prerequisite for the study of the isolated Hb β-chain is the purification from β_1 components At present, for this purpose cation-exchange chromatography of HbA_0 preparations must be carried out as a first step, which must then be followed by *p*-hydroxymercuribenzoate (*p*-HMB) Hb chain separation, ion-exchange chromatography and final procedures for the removal of *p*-HMB. The overall procedure is long and strains the protein, which may therefore become denatured In order to overcome this problem, studies on isolated haemoglobin β-chain are often performed on samples that have not been previously purified

In order to shorten the time required for β-chain purification, we developed a procedure that employes a unique chromatographic step for the separation of $\beta_0^{p\text{-HMB}}$ from $\beta_1^{p\text{-HMB}}$. This technique not only allows the rapid preparation of pure isolated β_0-chain, but through some modifications it also provides a rapid separation of acceptably pure isolated β_{1c}-chain

EXPERIMENTAL

All common reagents of analytical-reagent grade were obtained from Merck (Darmstadt, F R G) and Carlo Erba (Milan, Italy) Bio-Rex 70 (200–400 mesh, Na^+ form) was from Bio-Rad Labs (Richmond, CA, USA), sodium *p*-HMB from Aldrich (Steinheim, F R G) and YM10 membranes from Amicon (Lexington, MA, U.S A)

Preparation of haemoglobin samples

Venous blood from donors was collected in EDTA-containing tubes. The erythrocytes were centrifuged at 1000 *g* for 10 min at 4 °C and then washed four times with phosphate-buffered saline [PBS, 20 mmol/l sodium phosphate (pH 7 4), 0 145 mol/l sodium chloride] The lysis was performed with two volumes of doubly distilled water per volume of packed erythrocytes at 4 °C for 30 min One volume of carbon tetrachloride was then added and the solution centrifuged at 1500 *g* at 4 °C for 15 min The clear supernatant was employed directly for the separation of α- and β-chains according to the *p*-HMB splitting method suggested by Bucci and Fronticelli [3], with a *p*-HMB/Hb molar ratio of 8 1 and a terminal pH of the titration of 5 9 The *p*-HMB preparation was left overnight at 4 °C, then centrifuged at 1500 *g* at 4 °C for 30 min to remove the massive precipitate The solution, containing α- and β-*p*-HMB chains, was dialysed against the buffer used for the following cation-exchange separation

Cation-exchange chromatography on Bio-Rex 70

Prior to column preparation, Bio-Rex 70 was equilibrated with 0.028 *M* potassium phosphate buffer (pH 6 50) for β_0 preparation and with 0 016 *M* potassium phosphate buffer (pH 6 45) for β_{1c} preparation The equilibration was usually carried out in a large column using, over several days, extensive vol-

umes of buffer, until the pH reached the desired value After the equilibration, the resin was transferred to the separation column (90×1 6 cm I D) and, after further controls of the pH of the eluted buffer, the sample was loaded (about 180 mg of total Hb for 50 g of resin) The flow-rate was 1 2 ml/min and the fraction volume was 4 0 ml The fractions were analysed spectrophotometrically using a Lambda 5 UV–VIS instrument (Perkin-Elmer, Norwalk, CT, U S A) at 415 and 280 nm The fractions corresponding to the peaks of interest were pooled, concentrated on YM 10 membranes and analysed by isoelectric focusing (IEF) and by tryptic high-performance liquid chromatographic (HPLC), peptide mapping

Isoelectric focusing of globin chains

IEF was carried out according to the previously described method [4] on a polyacrylamide gel rod in 8 0 *M* urea and 2-mercaptoethanol using a mixture of two Ampholines (respective pH ranges 6–8 and 7–9) The prefocusing and focusing times were 30 and 210 min, respectively The gels were fixed by trichloroacetic acid and 5-sulphosalicylic acid and stained with Coomassie Brillant Blue R-250

Globin and Hb chain trypsinization and HPLC mapping

Both globins and isolated Hb chains, obtained directly from cation-exchange chromatography, were trypsinized according to the method of Schroeder et al [5]. The tryptic digest was analysed by reversed-phase HPLC mapping using a Millipore-Waters (Milford, MA, U S.A.) chromatographic station equipped with two Waters 510 pumps, a 712 WISP automatic injector, a Lambda Max 481 LC spectrophotometer and Baseline 810 chromatographic software Eluent A was 20 m*M* sodium phosphate buffer (pH 5 80) and eluent B was 50% (v/v) acetonitrile in buffer A The gradient applied was from 0 to 100% B in 50 min, the flow-rate was 1 0 ml/min, the detection sensitivity was 0 2 a u.f s at 214 nm. The sample volume was 5 μl, equivalent to 250 μg of whole tryptic digest.

Removal of p-hydroxymercuribenzoate

The removal of *p*-HMB was carried out by a gel permeation on a Sephadex G-25 Superfine column (90×1 6 cm I D) equilibrated with 100 m*M* Tris–HCl buffer (pH 7 5) 1 m*M* in EDTA and 1 m*M* in 2-mercaptoethanol, after dialysis for 30 min against the same buffer, the sample was adjusted to the desired conditions by means of a Sephadex G-25 Superfine gel permeation column equilibrated with the buffer used in the following experiments

RESULTS

The cation-exchange chromatography traditionally used for the separation of $\beta^{p\text{-HMB}}$- from $\alpha^{p\text{-HMB}}$-chains is carboxymethylcellulose Since all attempts to obtain a direct separation of $\beta_1^{p\text{-HMB}}$ from $\beta_0^{p\text{-HMB}}$ with this resin failed, we decided to use cation-exchange chromatography with Bio-Rex 70 The use of a low pH together with an elution buffer of low ionic strength provided a good separation of β-chains In Fig. 1a the chromatographic pattern obtained at pH 6 50 with a potassium phosphate buffer is shown The separation was particularly aimed at $\beta_0^{p\text{-HMB}}$ purification; the peaks obtained were pooled and analysed by IEF in urea and 2-mercaptoethanol, in order to verify the component purity, as represented in Fig 2 2-Mercaptoethanol cleaves the bond between p-HMB and cysteine and the globin bands can be compared directly with normal whole Hb samples. From the IEF patterns, the first chromatographic peak obtained was found to include all β_1 components, and the second peak corresponded to a unique IEF band at an apparent isoelectric point (pI_{app}) more acidic than that expected from the chromatographic elution; from subsequent tryptic digestion, the latter peak was identified as a β-chain (Fig 4, b3) We

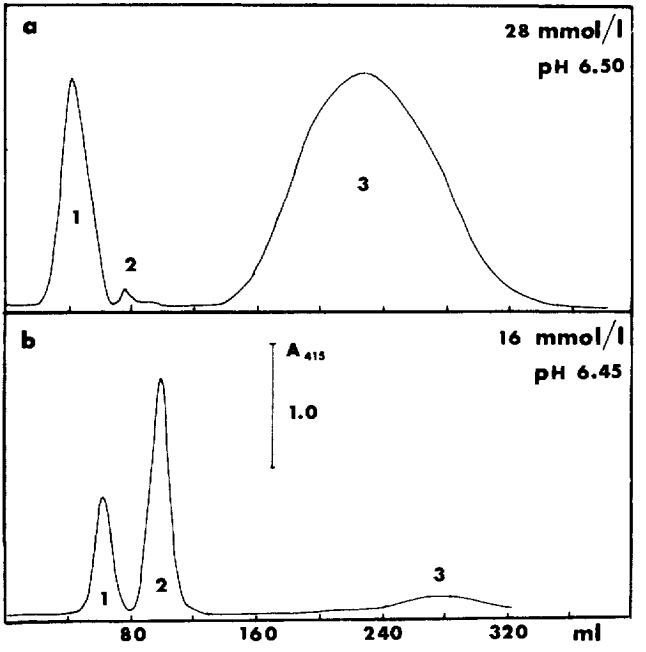

Fig 1 Cation-exchange chromatography of $\beta^{p\text{-HMB}}$ chain on Bio-Rex 70 (a) Pattern obtained using 28 mM potassium phosphate buffer (pH 6 50), (b) pattern obtained using 16 mmol/l potassium phosphate buffer (pH 6 45) The numbers on the peak correspond to the pools collected Pool 3 in (b) is equivalent to pool 2 in (a)

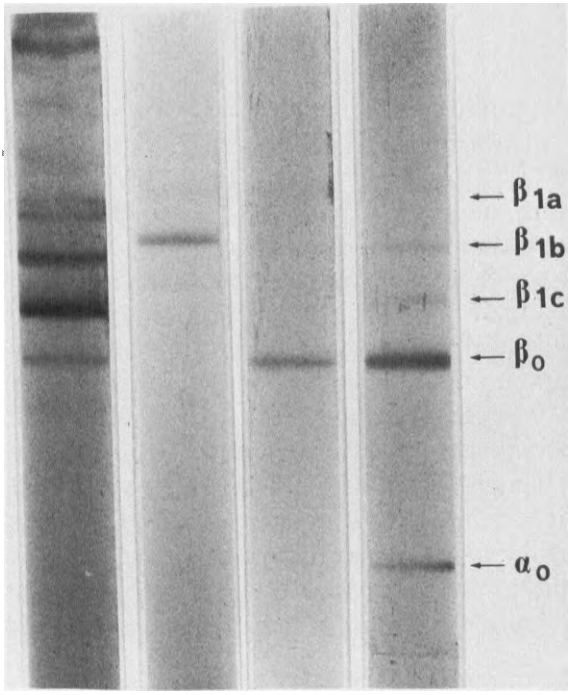

Fig 2 IEF pattern of peaks 1, 2 and 3 in Fig 1a The fourth lane shows the IEF pattern of whole Hb sample

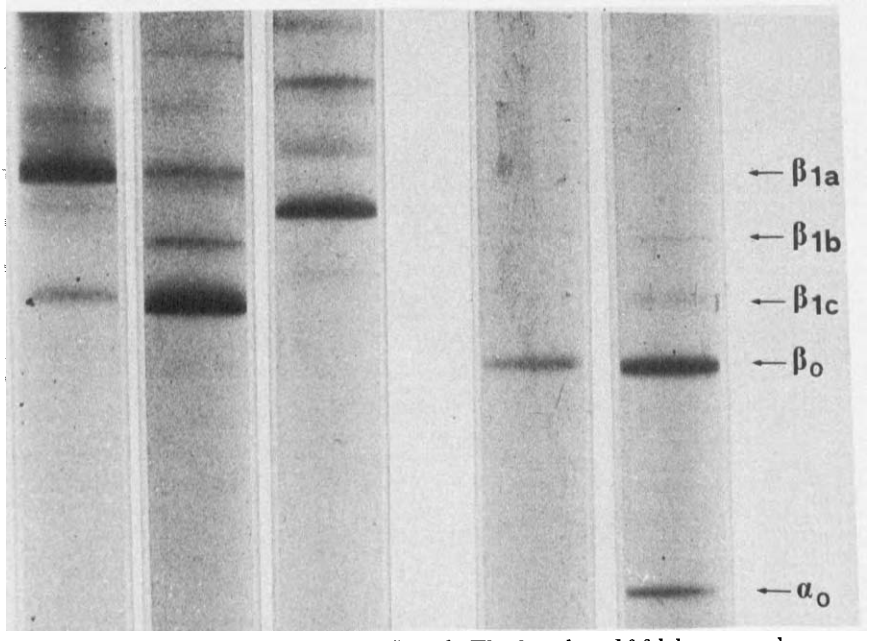

Fig 3 IEF pattern of peaks 1, 2 and 3 in Fig 1b The fourth and fifth lanes are shown, as reference, for β_0 and whole Hb samples, respectively

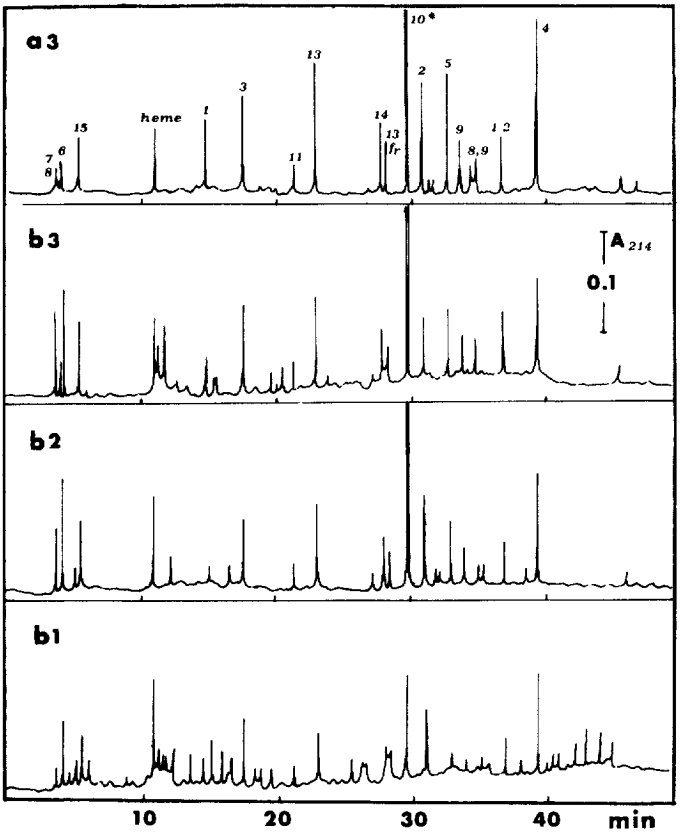

Fig 4 Reversed-phase HPLC patterns of β-chain tryptic digests from some peaks obtained by means of cation-exchange chromatography on Bio-Rex 70 as shown in Fig 1 The number used for each HPLC pattern refers to the peak, and the letter corresponds to the panel in Fig 1 In pattern a3 the identification number of the β-chain tryptic fragments is indicated (i e , number 4 corresponds to βT_4 fragment), 13_{fr} indicates an anomalous tryptic fragment from βT_{13}, the notations 8, 9 and 1, 2 indicate uncleaved fragments, 10* indicates the βT_{10} fragment modified on Cys_{93} by *p*-HMB

were not able to detect the modification accounting for these inconsistent results The third peak contained the highly pure β_0 component In the IEF pattern, in addition to the β_0 bands, two tiny bands corresponding to the pI_{app} of the β_{1b-c}-chains are present Their very low percentages indicate that they probably originate from lysinic glycation [6] In any case, the purification of β_0 is adequate and rapid

In order to separate β_1 components, the pH and ionic strength were decreased using a 0 016 *M* potassium phosphate elution buffer (pH 6 45). In this case (Fig. 1b), the delay in the elution allows the separation of only $\beta_1^{p\text{-HMB}}$ to be obtained promptly, whereas $\beta_0^{p\ HMB}$ is obtainable within a reasonable time

only with the application of a gradient. We therefore preferred to use this chromatography only for the separation of β_1 components The IEF pattern (Fig. 3) shows that, in this chromatography, the second-eluting peak includes primarily isolated β_{1c}-chain with slight contamination from β_{1a-b} components, which are essentially contained in the first chromatographic peak

Both the reversed-phase HPLC of $\beta_0^{p\text{-HMB}}$ tryptic digest obtained from the first chromatography and that of $\beta_1^{p\text{-HMB}}$ digest obtained from the second chromatography are shown in Fig. 4 In all the HPLC patterns the principal tryptic fragments of β-chains can be detected The tryptic fragment β_{10} (amino acids 83–96) is normally undetectable by HPLC mapping but, after the reaction of Cys_{93} with *p*-HMB, its solubility increases and it elutes as a highly absorbing peak, indicated by 10* in Fig 4

Whereas the HPLC patterns of β_0 (Fig. 4, a3) and β_{1c} (b2) indicate complete isolation, the pattern derived from β_{1a-b} (b1) shows substantial contamination due to the presence of unidentified peaks that do not seem to originate from β-chain fragments. Fig. 4 (b3) shows the HPLC pattern of the tryptic digest relative to the third peak of the second chromatography, which corresponds to the second peak of the first cation-exchange chromatography on Bio-Rex 70, as deduced from the IEF patterns The HPLC pattern in Fig 4 (b3) clearly shows a series of peaks corresponding to some of normal β_0-chain tryptic fragments, together with further unidentified peaks that could be derived either from post-translational modification of lateral amino acid chains or from some undetectable protein contaminants.

CONCLUSIONS

Cation-exchange chromatography on Bio-Rex 70 allows a good separation of β_0 component without β_1 contamination; further, by changing the pH and ionic strength of the eluent employed, a satisfactory preparation of pure β_{1c} is obtainable In this study we preferred to use two different sets of conditions in order to achieve these two aims; nevertheless, the employment of a suitable gradient might ensure the simultaneous separation of both components The chromatographic patterns obtained under both sets of conditions show that three components can be obtained from β-chain splitting, viz., β_{1a}, β_{1b} and β_{1c}, although double IEF bands are detectable in the β_{1a} and β_{1b} components (Fig. 2) This suggests, as previously hypothesized [4], that more than one chemical modification could provide similar changes in pI_{app} and similar chromatographic properties These results indicate that the β modifications, involved in the generation of β_1 components, are limited to a few kinds glycation, which provides β_{1c}, and the puzzling modifications that supply β_{1a} and β_{1b} components, some workers have in fact suggested [7, 8] several different interpretations of their structures, nevertheless, and considering our results, their complete identification remains unsatisfactory In any instances the

chromatographic pattern obtained from β-chains is much simpler than that expected from cation-exchange chromatography of HbA; the latter recently allowed the detection of at least nine different components [4,9,10]

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