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Hb-LINKÖPING (β36 Pro → Thr): A NEW HEMOGLOBIN MUTANT CHARACTERIZED BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Preparative separation of α- and β-chains from a newly identified abnormal hemoglobin has been performed on a new C_1/C_8 column. Tryptic peptides from the abnormal β-chain were subjected to peptide mapping on a new C_2/C_{18} column with a reversed-phase system including potassium dihydrogen phosphate (pH 2.9) as a hydrophilic ion-pairing reagent. A hydrophobic substitution, β36 proline–threonine, was evident after amino acid analysis and Edman degradation of the isolated mutant peptide. Replacement of proline as the second amino acid in the C-helix represents an important structural change in the $\alpha_1\beta_2$-contact.

INTRODUCTION

The possibility of detecting structural differences between normal and abnormal hemoglobins is of significant clinical value. Approximately half of the 350 known hemoglobinopathies represent (a) mutations that disrupt the tertiary structure leading to unstable hemoglobins, manifested clinically as haemolytic anemia; (b) mutations affecting the $\alpha_1\beta_1$- and $\alpha_1\beta_2$-contacts that influence the conformation of the tetramer, which may result in increased or decreased oxygen affinity; (c) M-hemoglobins with cyanosis; and (d) aggregating hemoglobins (HbS).

Many hemoglobin variants of clinical interest have slightly different p*I* values, as shown by electrofocusing, which indicate hydrophobic substitutions. Reversed-phase high-performance liquid chromatography (RP-HPLC) has been suggested the separation for both the globin chains[1,2] and their tryptic peptides[3–5], because the separations based on differences in hydrophobicity.

The purpose of this investigation was to show the separation capacity of new bonded-phase silica gels[6] by characterizing a hemoglobin mutant that is electrophoretically silent. The patient studied, who was of Finnish origin, manifested clinical symptoms of a polycythemia due to an increased oxygen afbinity of the abnormal hemoglobin.

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MATERIALS AND METHODS

Chemicals and reagents

EDTA-treated blood was collected and transported to the laboratory within 24 h at room temperature. A 1-ml volume of packed red cells was washed three times with 10 ml of saline and, after a 5-min centrifugation at 3000 *g*, the supernatant was discarded. To induce hemolysis, 2.0 ml of water and 0.5 ml of carbon tetrachloride were added. After centrifugation, the clear supernatant was free of stroma.

Sodium dihydrogen phosphate, orthophosphoric acid and trifluroacetic acid were AristaR grade reagents from BDH. Acetonitrile (HPLC grade) was obtained from Blackford, Wells Limited, U.K.

Equipment and chromatographic conditions

A Pharmacia FPLC system, including two P-500 pumps, a GP-250 gradient programmer and a 214-nm UV detector, was used for all chromatographic separations. Analytical and preparative separations of globin chains were performed by RP-HPLC on a Pharmacia HR 5/10 Pro RPC prepacked column (C_1/C_8, 5 μm particle size, 300 Å pore-size support). Elution was performed with a 60-min linear gradient from 0 to 50% B at a flow-rate of 0.2 ml/min. Solvent A was 39% acetonitrile–0.3% trifluoroacetic acid, and solvent B, was 50% acetonitrile.

A 50 μl (0.25 mg) volume of tryptic peptides was directly injected onto a Pharmacia HR 5/5 Pep RPC prepacked column (C_2/C_{18}, 5 μm particle size, 100 Å pore size). Elution was performed with an 80-min linear gradient from 0 to 75% B at a flow-rate of 0.7 ml/min. Solvent A was 49 m*M* potassium dihydrogen phosphate, pH 2.9, in water, and solvent B was acetonitrile–solvent A (50:50)[7].

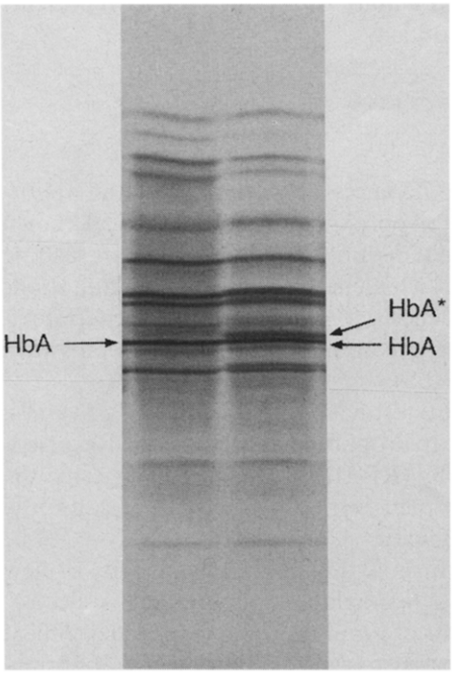

Fig. 1. Isoelectric focusing of normal (A) and mutant (A*) hemoglobins in the pH interval 3–10.

Experimental

Isoelectric focusing[8] and 2-propanol stability tests[9] were performed as described earlier. Tryptic digestion of isolated, freeze-dried globin chains (5 mg/ml) in 0.1 *M* ammonium hydrogen carbonate was carried out at 37°C for 3 h with a 5% (w/w) solution of TPCK-treated trypsin.

Amino acid analyses of the collected peaks were performed after hydrolysis in 6 *M* hydrochloric acid at 110°C under vacuum for 24 h. A Beckman 890C automatic sequencer with the 1 *M* Quadrol-Polybrene system was used for sequence determinations.

RESULTS

Isoelectric focusing on Pharmalyte 3-10 indicated that the patient was a heterozygous carrier for an abnormal hemoglobin (Fig. 1). The patient was a non-smoker and had a hemoglobin value slightly above the reference value: 180 (130–160) g/l. The presence of a high-affinity hemoglobin was confirmed by oxygen equilibrium

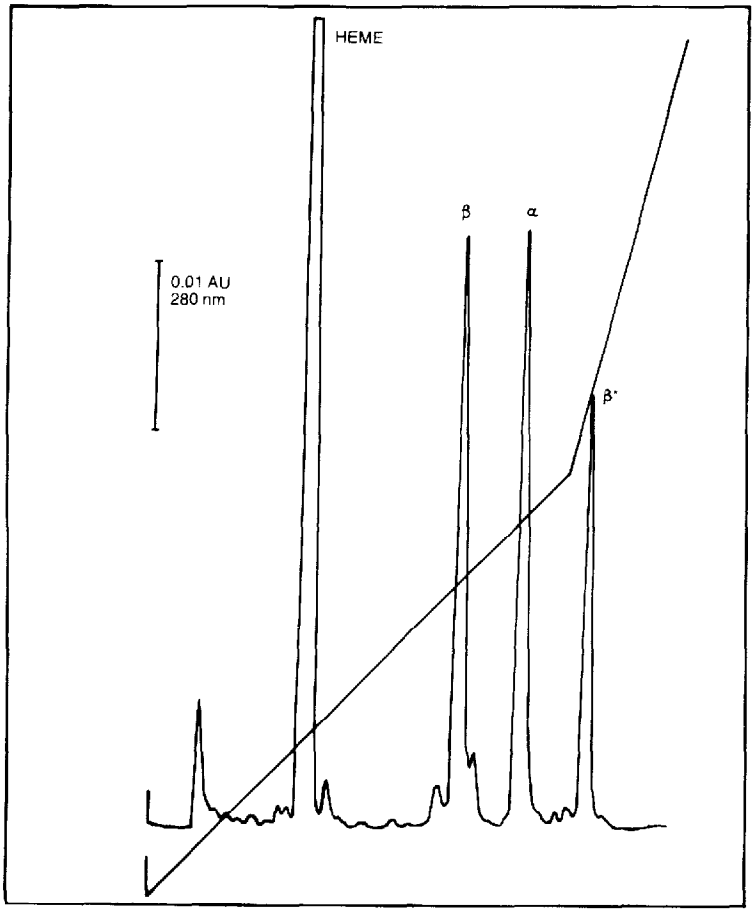

Fig. 2. Globin chain separation on a Pro RPC HR 5/10 column. β^* and β indicate the mutant and normal β-chains, respectively.

measurements. A P_{50} value of 18 mmHg (2.2 kPa) was obtained, compared with a control value of 26 mmHg (3.5 kPa). The 2-propanol test also indicated a slightly unstable hemoglobin. Analysis of the hemolysate by RP-HPLC on a C_1/C_8 column revealed the presence of both a normal and an abnormal β-chain (Fig. 2), which confirmed the result obtained by isoelectric focusing. The yield of the abnormal β-peak was slightly reduced as some material had been lost during storage and preparation of the unstable fraction. The material from 1.4 mg of hemoglobin was sufficient for RP-HPLC analysis of the tryptic digest of the two β-chains. The mutation was located in T4 (Fig. 3), containing residues 31–40 (part of C-helix) of the intact β-chain. The T4-peptide was missing in the peptide mapping of the β*-chain. Amino acid analysis and sequence degradation of the T_4* peptide (Table I) gave the composition Leu–Leu–Val–Val–Tyr–*Thr*–Trp–Thr–Glu–Arg, indicating the replacement of proline (β36) with a threonine residue. Two smaller fragments, which also appeared in the chromatogram, were cleavage products of T4* (a = N-terminal, b = C-terminal) after unexpected rupture of the Tyr–Thr bond. This tryptic attack explains the low yield of T4*.

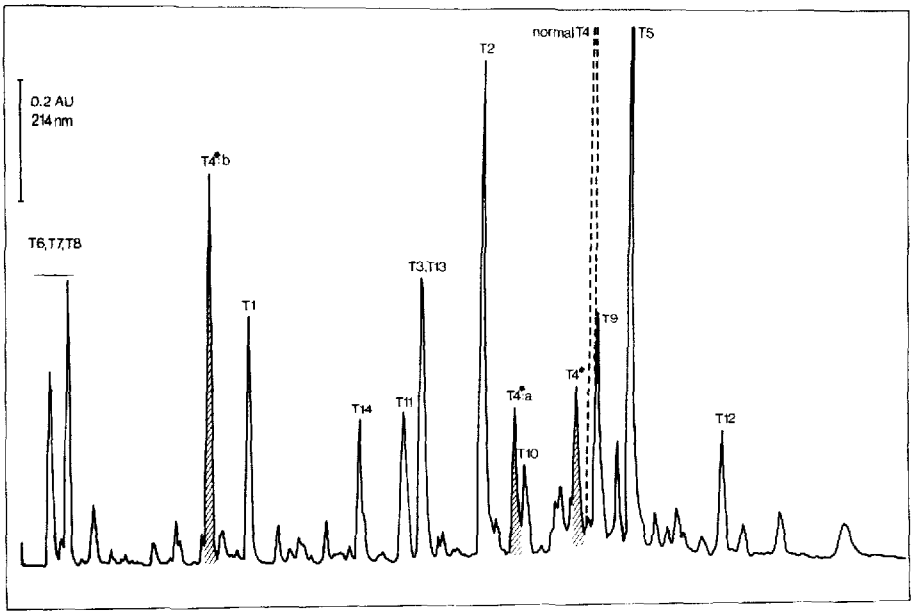

Fig. 3. Separation of a tryptic digest of the mutant β-chain on a Pep RPC HR 5/5 column. Asterisks indicate peptides deviating from the normal tryptic pattern. The elution position of the normal T4 peptide is indicated.

DISCUSSION

This case represents an "electrophoretically alomost silent" mutant with a hydrophobic substitution β36 Pro-Thr in the important part of the C-helix. With previous techniques, such as chromatography on CM-cellulose with 6 *M* urea and the fingerprint technique, which resulted in low preparative yields it was troublesome to

TABLE I

AMINO ACID COMPOSITIONS OF THE NORMAL TRYPTIC PEPTIDE T4, THE MUTANT PEPTIDE T4* AND THE TWO CLEAVAGE PRODUCTS T4a AND T4b

Amino acid	*T4*	*T4**	*T4*a*	*T4*b*
Threonine	0.9	1.8	–	1.8
Glutamic acid	1.0	1.0	–	1.0
Proline	0.9	–	–	–
Valine	1.9	1.9	1.9	–
Leucine	2.0	2.0	2.0	–
Tyrosine	0.8	0.8	0.8	–
Tryptophane	*	*	–	*
Arginine	0.9	0.9	–	0.9

* Not determined; indicated by the UV absorbance at 280 nm.

examine such cases. During 1979 several investigators reported the separation of normal and abnormal hemoglobin chains by HPLC[1,2]. Hemolysates were in our case diluted in solvent A and applied after *ca.* 30 min when the heme was dissociated and the β- and α-chains were disaggregated.

Corresponding eluates were freeze-dried before tryptic digestion. A small aliquot (100 μg) of tryptic peptides in buffer could be directly applied to the C_2/C_{18} column. A preparative chromatogram of 500 μg was sufficient for both the amino acid analysis and sequence studies of a single peptide. Several solvent systems have been carefully tested for tryptic peptide separation on C_{18} columns[5]. So far, we have obtained the best results with the potassium dihydrogen phosphate system on our column. All tryptic peptides of the β-chain were separated, except T3 and 13. The results presented here indicate that the core peptides, Tp 10, 11 and 12, can be separated and recovered in high yields. This has not been possible previously by means of the fingerprint system. Our method eliminates the chemical modification of cysteine to form the aminoethyl analogue of lysine for the introduction of additional tryptic hydrolysis sites. The reagent ethyleneimine has carcinogenic properties. The phosphate–acetonitrile system has the disadvantage of involatility of phosphate. However, it does not interfere with amino acid analysis or Edman degradation. Peaks can be monitored at 206 or 214 nm and simultaneously at 254 or 280 nm to identify peptides containing tryptophan, tyrosine or phenylalanine.

The physiological effect of the substitution can be explained from the work of Perutz[10]. β36 Pro, the second amino acid in the C-helix, is well conserved during evolution. The C-helix represents the major part of the nineteen amino acids in the $\alpha_1\beta_2$-contact. The Pro 36 helps to form the corner between the B and C helices. Thus, the substitution by threonine interferes with the tertiary structure of the chains and thereby alters the oxygen affinity.

NOTE ADDED IN PROOF

An abnormal hemoglobin with the same amino acid substitution β36 Pro → Thr has been found in Finland; patients were unrelated (Y. Wada and H. Lehmann, unpublished results).

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