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Structural Characterization of Hemoglobin Tacoma*

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ABSTRACT: Chemical characterization of hemoglobin Tacoma demonstrates a single substitution of a seryl residue for the arginyl residue number 30 of the β chain. Because this appears to be the only substitution in the abnormal hemoglobin, the structural formula is $\alpha_2\beta_2{}^{30\text{Ser}}$. This substitution occurs in a region of residues 28–42 which is one of the relatively invariant parts of the β chain of normal animal hemoglobins.

Baur and Motulsky (1965) have described a new inherited hemoglobin variant, Hb Tacoma, which they discovered in three healthy members of a family of European origin. The proportion of the abnormal hemoglobin in the heterozygote was found to be 43% of the total hemoglobin. Clear separation of Hb Tacoma from Hb A has been achieved only by starch grain electrophoresis, where Hb Tacoma moves more rapidly towards the anode. Hemoglobin Tacoma is less heat resistant and is more rapidly denatured by 8 M urea than Hb A, Hb C, or Hb S. Hybridization experiments by Baur and Motulsky (1965) indicated the presence of a structural abnormality in the β chain and a normal α chain in Hb Tacoma.

The subject of this paper is the chemical study of the amino acid substitution in the abnormal β chain of Hb Tacoma.

Experimental Procedure

Isolation of Hb Tacoma from Hb A was by repeated starch block electrophoresis as described previously by Baur and Motulsky (1965). A small amount of the purified abnormal hemoglobin was hydrolyzed with trypsin according to the procedure of Ingram (1958). Approximately 2–3 mg of a freeze-dried preparation of this tryptic digest dissolved in 20 μl of 0.001 N HCl was applied to Whatman No. 3MM paper. Electrophoresis was carried out in a pyridine–acetic acid buffer of pH 6.5 for 2 hr at 2000 V (Baglioni, 1961). Ascending chromatography was effected with a solvent mixture of pyridine–3-methyl-1-butanol–water (7:7:6, v/v). The peptide patterns were reacted with ninhydrin and specific stains for arginine, tyrosine, histidine, and tryptophan residues (Lehmann and Huntsman, 1966).

Globin was prepared from most of the purified hemoglobin by removing the heme with cold acid acetone ac-

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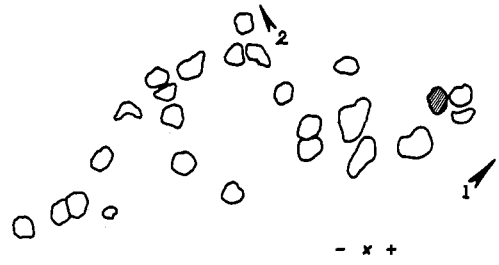

FIGURE 1: A schematic representation of the peptide pattern of a tryptic digest of Hb Tacoma by two-dimensional electrophoresis and chromatography on filter paper. The circles represent peptides which were detected by ninhydrin. The hatched area indicates the position of a new abnormal peptide βT3,4 which reacted for arginine, tyrosine, and tryptophan. Arrows 1 and 2 indicate the location of the normal peptides βT-3 and βT-4, respectively, which were absent in Hb Tacoma.

cording to the procedure of Anson and Mirsky (1930). The α and β chains were separated by column chromatography on carboxymethylcellulose using an ionic gradient in 8 M urea according to the method of Clegg *et al.* (1966). The β chains were treated with ethylenimine (Jones, 1964; Clegg *et al.*, 1966) in order to produce the *S*-aminoethyl derivative. Tryptic hydrolysis of the *S*-aminoethyl β chain was effected with a 1:100 weight ratio of enzyme (Worthington twice-crystallized, salt-free trypsin) to protein in a solution buffered with trimethylamine at pH 8–9 for 2–4 hr at room temperature according to the procedure of Baglioni (1965).

For preparative purposes the tryptic peptides of 20–30 mg of *S*-aminoethyl β chains were isolated and purified by automatic peptide chromatography on columns of ion-exchange resins. The peptides were separated initially on a 0.9 × 17 cm column of either Spinco 15A or Bio-Rad Aminex A-5 cation-exchange resin at 50° using a linear gradient of pyridine–acetic acid developer beginning at pH 3.1, 0.2 M in pyridine and ending at pH 5.0, 2.0 M pyridine at a flow rate of 30 ml/hr. The developer gradient was formed by using two cylinders of equal diameter joined by a connecting tube at their bases. At the beginning, the mixer was filled with 250 ml of the pH 3.1, 0.2 M pyridine–acetic acid buffer and the reservoir was filled with 250 ml of the pH 5.0, 2.0 M pyridine–acetic acid buffer. The compositions of these buffers are given by Schroeder *et al.* (1962). A portion of the effluent, generally $^1/_{20}$ of the total, was separated by a stream divider pump and continuously reacted with ninhydrin in order to detect the position of the peptides. The details of this automatic peptide chromatography have been presented elsewhere (Jones, 1964, 1967). Most peptides were further purified by rechromatographing each zone on a 0.9 × 60 cm column of Bio-Rad AG 50W-X2 (270–325 mesh) with the same developer gradient beginning at pH 3.1 and ending at pH 5.0 that was used for the preparative chromatograms with the columns of Spinco 15A resin. For the rechromatography of some peptides, an anion-exchange column (0.6 × 60) cm containing Bio-Rad AG 1–X2 was used with a linear gradient consisting of 100 ml of 1% collidine adjusted to pH 8 with acetic acid and 100 ml of 1.0 M acetic acid (Guest *et al.*, 1967). This was followed by 1.0 M acetic acid. A flow rate of 30 ml/hr was used for rechromatography with one-fifth of the effluent being used for reaction with ninhydrin.

Portions of the isolated peptides were hydrolyzed for either 22 or 72 hr at 110° in evacuated, sealed-glass vials in 6 N HCl containing 9 mg % phenol. Quantitative amino acid analyses were made with a Spinco Model 120 amino acid analyzer modified to include a 20-mm long-path photometer flow cell (Jones and Weiss, 1964).

Chemical cleavage of peptides at methionine residues was effected by treating about 0.1 μmole of peptide in 1 ml of 0.1 N HCl with 1–2 mg of cyanogen bromide over a period of 24 hr at 40° (Chu and Yasunobu, 1964). The reaction mixture was then dried under reduced pressure with a rotary evaporator. The residue was dissolved in the starting pyridine–acetic acid developer at pH 3.1 and chromatographed on a column of Bio-Rad AG 50W-X2.

Results

A peptide pattern of the tryptic hydrolysate of Hb Tacoma which was obtained on filter paper was stained for arginyl peptides as illustrated in Figure 1. The peptides which were detected by prior staining with ninhydrin have been circled. This pattern differs from patterns for Hb A in that the normal βT-3 and βT-4 peptides were not detectable and a new peptide was found anodal to the point of application and just towards the neutral side of βT-5. This new peptide was virtually unstained by ninhydrin but gave reactions for tryptophanyl and tyrosyl as well as arginyl residues.

A peptide pattern by automatic column chromatography is shown in Figure 2 for the tryptic hydrolysate of 26 mg of S-aminoethylated β chain of Hb Tacoma. This pattern is very similar to that obtained for Hb A except that zone I is larger than normal and zones II and VI are smaller than normal (for example, see Figure 5 of Jones, 1967). It was only after rechromatography of each zone from this and similar chromatograms that the abnormalities were established. The normal βT-3 and βT-4 peptides were found to be absent from their usual positions in zones II and VI of Figure 2. The normal βT-13 peptide was present and accounts for most of zone II which appeared in this chromatogram. Similarly, the normal βT-14 peptide accounted for most of zone VI. The tripeptide Thr-Gln-Arg, which results from a partial "chromotryptic-like" cleavage of the C-terminal region of the βT-4 peptide was found in low yield as usual in zone XII.

The portion of the β chain accounting for the missing βT-3 and βT-4 peptides was located in zone I (shaded area) of Figure 2. Rechromatography of this zone on Bio-Rad AG 50W-X2 gave a zone whose amino acid composition suggested a mixture of βT-3, βT-4, and βT-5. In spite of several different chromatographic conditions, the removal of βT-5 from this zone was ineffective until a portion of zone I was treated with cyanogen bromide and then rechromatographed on Bio-Rad AG 50W-X2. This resulted in the resolution of zone I

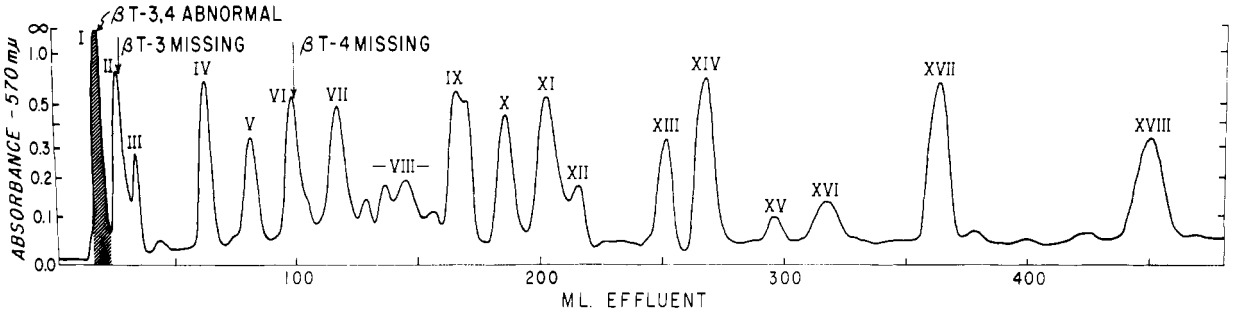

FIGURE 2: Peptide pattern of tryptic hydrolysate of the aminoethylated beta chains from Hb Tacoma. The shaded zone contains the abnormal peptide.

into three peptide peaks. The two peptides resulting from the cleavage of βT-5 at its methionine residue were eluted at new positions while the remaining peptide material chromatographed as originally noted. Amino acid analysis of this latter zone resulted in a composition corresponding to equimolar amounts of βT-3 and βT-4 with a serine residue replacing one of the arginine residues. However, this peptide was still contaminated by approximately 15% of unreacted βT-5.

A better separation was achieved by chromatographing a new tryptic hydrolysate of 25 mg of aminoethylated β chain on a column of Aminex A-5 resin using a pH 2.5, 0.05 M pyridine–acetic acid buffer containing 4 ml of pyridine and 300 ml of acetic acid per l. This buffer was run for 60 ml before beginning a linear gradient obtained from 250 ml of this buffer in the mixer and 250 ml of a pyridine–acetic buffer of pH 3.5. The latter was prepared by adding pH 5.0, 2.0 M pyridine–acetic acid buffer to pH 3.1, 0.2 M pyridine–acetic acid buffer until the pH is 3.5. Although most of the tryptic peptides are not eluted under these conditions, the abnormal βT-3,4 was eluted at the front free of βT-5. The abnormal zone was purified from other minor contaminants by rechromatography on an anion-exchange column of Bio-Rad AG 1-X2. The amino acid composition of the pure peptide, which is shown in Table I, confirms the previous finding of a seryl residue in place of one of the arginyl residues. Also shown in Table I are the amino acid compositions of all of the other tryptic peptides of the β chain of Hb Tacoma. The observed values agree with those expected for normal tryptic peptides of the β chain.

Discussion

It is reasonable to conclude from the foregoing results that the arginyl residue number 30 of the normal β chain has been replaced by a seryl residue in Hb Tacoma.

18 βT-3 30
ValAsnValAspGluValGlyGlyGluAlaLeuGly*Ser*Leu-
βT-4 40
LeuValValTyrProTryThrGlnAgr

Such an amino acid substitution would prevent the normal tryptic hydrolysis at residue 30 which gives rise to the separate βT-3 and βT-4 peptides. In addition, the presence of normal βT-5 and the detection of the tripeptide Thr-Gln-Arg which corresponds to residues 38, 39, and 40 of the β chain indicates that arginyl residue 40 is normal in Hb Tacoma. Further sequence determination was not done.

The portion of the β chain comprising residues 28–42 is considered to be a relatively invariant part of the molecule when one compares the β, δ, and γ chains of human and several other animal species (Schroeder and Jones, 1965). Three abnormal hemoglobins are known to be affected in this region. These are Hb Genova, β 28 Leu→Pro (Sansone *et al.*, 1967), Hb Hammersmith, β 42 Phe→Ser (Dacie *et al.*, 1967), and Hb Philly, β 35 Tyr→Phe (reported in Perutz and Lehmann, 1968). These abnormal hemoglobins are chemically unstable and are associated with hemolytic anemia. Although Hb Tacoma has been found to be denatured more readily than normal Hb A by heating, it is not associated with any detectable clinical abnormality in the heterozygous individual (Baur and Motulsky, 1965). Sansone *et al.* (1967) attribute the instability of Hb Genova to a disruption of the normal B helix of the β chain which results from the substitution of a prolyl residue for the normal leucyl residue β 28. Similarly, Dacie *et al.* (1967) conclude that the instability of Hb Hammersmith is due to the loss of the phenylalanyl residue 42 which normally stabilizes the heme group by van der Waals contacts. The seryl group which has been substituted in Hb Hammersmith presumably no longer interacts properly with the heme group and this permits distortion of the β chain leading to a decrease in the stability of the hemoglobin.

The recently published studies by Perutz *et al.* (1968) of the 2.8-Å resolution of hemoglobin indicate that the normal arginyl residue 30 of the β chain interacts with two residues of one of the α chains. This arginyl residue undergoes van der Waals interaction with histidyl residue α 122 and hydrogen bonding with phenylalanyl residue α 117. The substitution by a seryl residue in Hb Tacoma probably leads to a loss of some or all of these stabilizing effects. Such a loss could account for decreased stability of Hb Tacoma to heat and concentrated urea. The internal location of residue 30 of the β chain shields its side chain from the external solvent and must account for the unexpectedly small difference in net charge of Hb A and Hb Tacoma as detected by electrophoresis and ion-exchange chromatography. In most other abnormal hemoglobins in which a positive amino

TABLE 1: Amino Acid Analysis of Tryptic Peptides of AE β Chain of Hb Tacoma[a]

	IX[b] βT-1	VII βT-2	I βT-3,4 Abnormal	Theorem for Normal βT-3, βT-4	I βT-5	XIII βT-6	XVII βT-7	IX βT-8	V βT-9	XI βT-10	X βT-11	IV βT-12a	XVIII βT-12b	II βT-13	VI βT-14	XIV βT-15
Tryptophan		tr	0.09[c]	1												
Lysine	1.00	1.02			1.05	1.03	0.87	1.00	1.00	1.05			1.02	0.93	1.01	
AE-Cysteine										0.78		0.77				
Histidine	0.89						0.92		1.01	0.99	1.10		1.95		0.99	1.08
Arginine			1.11	2							0.87					
Aspartic acid			2.13	2	3.03				3.00	1.08	2.01	1.03		0.13	0.98	
Threnonine	1.00	0.97	0.96	1	1.05					1.90				0.97		
Serine		0.94	1.02	0	1.80				1.00	0.92						
Glutamic acid	2.16		2.92	3	1.09					0.98	0.98			2.92		
Proline	1.00		1.09	1	1.90						1.10			2.17		
Glycine		1.05	2.91	3	1.92		1.08		2.01	0.97		1.00	1.02	0.13	1.06	0.13
Alanine		2.03	1.02	1	0.95		1.14		2.00	1.01		0.13	1.02	1.99	4.00	
Valine	0.97	0.98	4.83	5	1.02	0.97			1.01		0.92	1.92	0.95	1.03	2.70	
Methonine					0.66											
Leucine	1.07	1.01	3.09	3	1.10				4.05	2.02	1.01	3.05	0.98		1.00	
Tyrosine			0.91	1										0.95		0.92
Phenylalanine					2.92				1.01	0.97	1.01		1.00	1.05		

[a]Molar ratios of amino acids produced by hydrolysis with 6 N HCl containing 9 mg % pheno for 22 hr at 110° under reduced pressure. Peptides βT-3,4 and βT-14 were hydrolyzed 70 hr. Values less than 0.12 residue have not been included. [b]Zones from the chromatogram in Figure 2. Each zone has been purified by rechromatography. [c]This peptide gave a strong reaction for tryptophan when treated with Ehlich's reagent on filter paper. The low quantitative value for tryptophan is due to destruction of this amino acid by acid hydrolysis.

acid residue is substituted by a neutral residue, a change of from one to two charges has been detected.

Most unstable hemoglobins have been detected because of their association with hemolytic disease (Motulsky and Stamatoyannopoulos, 1968). Hemoglobin Tacoma was discovered as a result of screening of blood samples for evidence of electrophoretic abnormalities. The instability of Hb Tacoma in the absence of hematologic findings suggests that there may be other unstable hemoglobins without significant clinical manifestations. Studies on *in vitro* chemical lability, therefore, are indicated in all hemoglobin variants for better understanding of the relationship of structure to function in the hemoglobin molecule. Some of these relationships of structure to function which can be deduced from studies of mutant hemoglobins such as Hb Tacoma have been described recently by Perutz and Lehmann (1968).

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Isolation and Characterization of the Cyanogen Bromide Peptides from the $\alpha 2$ Chain of Rat Skin Collagen*

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ABSTRACT: The $\alpha 2$ chain of rat skin collagen was cleaved at methionyl bonds with cyanogen bromide. The digest was fractionated by ion-exchange and molecular sieve chromatography and six peptides were isolated in approximately equimolar amounts. Each peptide represents a unique portion of the $\alpha 2$ chain as shown by chromatographic properties, amino acid analysis, and molecular weight. Together they account for all the amino acids of the $\alpha 2$ chain and its molecular weight of about 95,000. The peptides include a tripeptide, a tetradecapeptide, a peptide of 30 residues, and three large peptides each of about mol wt 30,000.

For studies of the primary structure of the collagen molecule, it has been shown that cleavage of methionyl peptide bonds with CNBr is a very useful method (Bornstein and Piez, 1965, 1966; Bornstein *et al.*, 1966). The collagen molecule contains three polypeptide chains, each having a molecular weight of about 95,000. Two of the chains, the $\alpha 1$ chains, in collagens from higher animals appear to be identical while the third, the $\alpha 2$ chain, differs in amino acid composition and chromatographic behavior from the two $\alpha 1$ chains (see Piez, 1967). Butler *et al.* (1967) have reported the cleavage with CNBr of the seven methionyl peptide bonds in $\alpha 1$ from rat skin collagen. Eight unique peptides were obtained, which account for all the

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