*THE PRESENCE OF TWO MAJOR HEMOGLOBIN COMPONENTS IN AN INBRED STRAIN OF MICE**

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For a number of reasons, laboratory mice offer an excellent system for the study of genetic control of hemoglobin structure. Mice have a rapid generation time, and therefore, large populations inbred for generations are readily available. Previous investigations have shown that the hemoglobins of different strains of mice are not identical when investigated by paper electrophoresis,[1] column chromatography,[2] and "fingerprinting," following tryptic digestion of the separated α and β chains.[2, 3]

The hemoglobins of certain strains of mice display discrete and quantitative differences in solubility in phosphate buffer relative to the hemoglobin of strain C57BL/6.[4] Although genetic studies indicated that the "low-solubility" characteristic segregated with the gene determining the structure of the α chain,[5, 6] no differences could be found when the "fingerprints" of the α-chain tryptic peptides of C57BL/6 hemoglobin were compared to those derived from the α-chain tryptic peptides of "low-solubility" hemoglobin obtained from the strain SEC.[2] Differences were found, however, between "fingerprints" of the β-chain tryptic peptides of C57BL/6 and SEC hemoglobin.[2] Since the low solubility of SEC hemoglobin is presumably a phenotypic expression of the α-chain structural gene, one would expect to find differences in the amino acid compositions of the α chains which could be responsible for the different solubilities of the hemoglobins from these two strains of mice.

Clearly, an attempt to explain these solubility differences by investigation of differences in amino acid sequences was desirable. Our study was undertaken with this as the objective.

For this purpose, the α and β chains of the SEC hemoglobin have been separated, digested with trypsin, the fragments isolated by ion exchange chromatography, and compared by amino acid analyses to the compositions of the C57BL/6 α- and β-chain tryptic peptides.[7, 8] Where differences were found, sequence studies were carried out.

Contrary to the results of other investigations,[2] we have found no differences in the amino acid composition of the β-chain tryptic peptides of C57BL/6 and SEC mice, while two α-chain tryptic peptides from SEC had amino acid compositions which differed from those of C57BL/6. In one of these peptides a single amino acid replacement was found, but in the other peptide a single residue of asparagine appeared to have been replaced partly by serine and partly by threonine; consequently, we had to postulate the existence of two α chains which could not be separated by our methods. Sequence analysis confirmed that 50 per cent of the α chains contained serine and the other 50 per cent contained threonine at position 68.

Materials and Methods.—All mice used in this investigation were generously supplied by Dr. Elizabeth Russell of the Jackson Laboratories, Bar Harbor, Maine. Mice were bled after decapitation, and hemoglobin was prepared according to the

method of Hill *et al.*[9] Heme was separated from globin, and α and β chains were separated as reported previously.[8, 9] Pure α and β chains were digested with trypsin and tryptic peptides isolated according to the procedures used for C57BL/6 β chain.[8] Amino acid analyses of the purified peptides were performed at 110°C for 22 hr in 6 *N* HCl as described by Spackman, Stein, and Moore.[10] Tryptophan was determined spectrophotometrically.[11] Peptide αTp-IX[12] was digested with pepsin and peptic peptides separated according to the procedure of Konigsberg and Hill.[13] The Edman degradations of peptide αTp-IX and the peptic peptide were done according to the modified procedures reported for human hemoglobin.[13]

Results.—By use of the methods previously described,[8] all of the α- and β-chain tryptic peptides from SEC hemoglobin were isolated (Fig. 1). All lysine and arginine residues were accounted for in unique tryptic peptides (Tables 1 and 2). The sum of the amino acid compositions of all of the tryptic peptides was in good agreement with the amino acid composition calculated from whole α and β chains. The amino acid analyses of the β-chain tryptic peptides (Table 1) revealed no differences between homologous peptides from SEC and C57BL/6. On the other hand, amino acid analyses of the α-chain peptides revealed a substitution of threonine for aspartic acid or asparagine in peptide αTp-XIII (Table 2). It was noted that when peptide

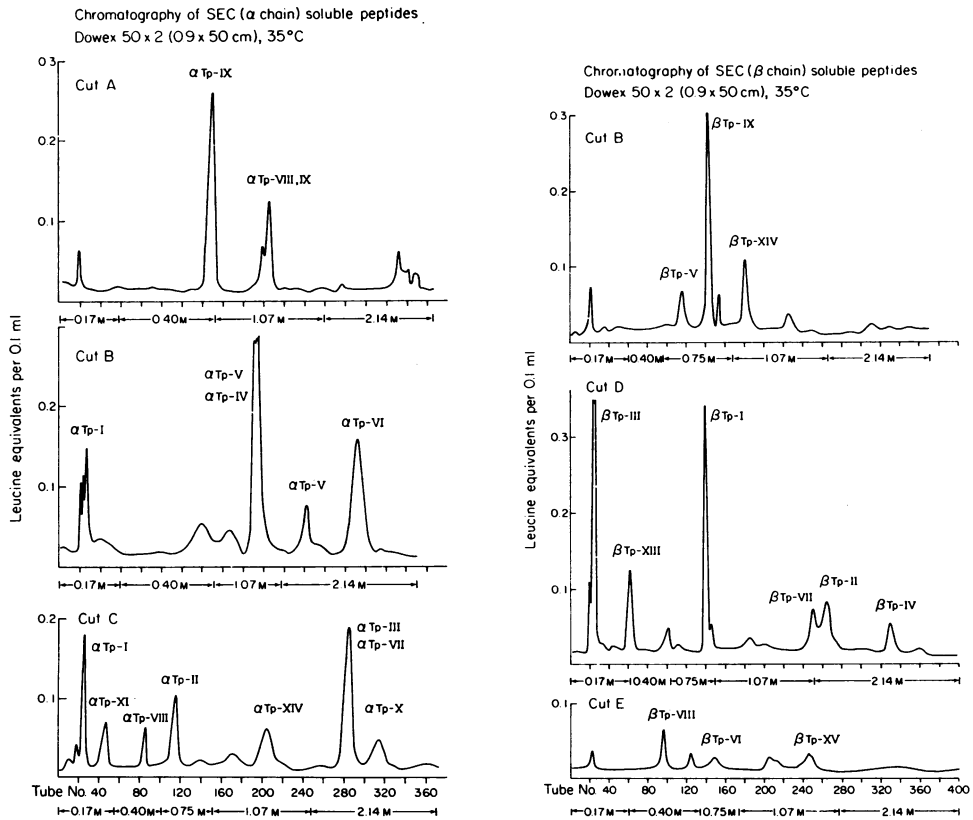

FIG. 1.—Chromatography of the soluble peptides from SEC α and β chains on Dowex 50 × 2 using pyridine acetate buffers[8] after preliminary fractionation on Sephadex G-25.[8]

TABLE 1

AMINO ACID COMPOSITIONS OF THE TRYPTIC PEPTIDES FROM C57BL/6 AND SEC β CHAINS

Amino acid	Amino Acid Residues per Molecule of Peptide															Sum of residues of tryptic peptides	Residues per β chain
	βTp-I	βTp-II	βTp-III	βTp-IV	βTp-V	βTp-VI	βTp-VII	βTp-VIII	βTp-IX	βTp-X	βTp-XI	βTp-XII	βpT-XIII	βTp-XIV	βTp-XV		
Lysine	1.13*	1.06	...	...	1.00	1.04	1.01	1.00	1.00	1.00	0.10	1.08	1.00	1.07	...		11.3
	1.01	1.00	...	...	1.00	1.03	0.95	1.00	1.00	1.00	0.20	0.96	1.00	1.07	...	11	10.7
Histidine	1.01	...	...	...	...	...	0.99	...	0.98	1.02	1.13	1.97	...	1.00	1.00		8.9
	1.03	...	...	...	...	...	1.02	...	1.12	1.00	1.04	1.91	...	0.95	1.08	9	8.6
Arginine	...	...	1.00	1.00	...	...	...	...	...	...	0.95	...	...	...	...		3.0
	...	...	1.00	1.00	...	...	...	...	...	...	1.16	...	...	...	...	3	2.8
Aspartic acid	1.01	...	1.92	...	3.10	...	...	...	4.08	1.12	2.13	1.26	0.99	...	...		15.3
	1.00	...	2.10	...	3.20	...	...	...	4.00	1.14	2.18	1.17	0.92	...	...	15	14.8
Threonine†	0.96	...	...	0.93	...	...	...	...	0.85	0.96	...	...	0.94	...	...		5.1
	0.96	...	...	0.80	...	...	...	...	0.97	0.90	...	...	1.01	...	...	5	4.6
Serine†	...	0.96	...	...	3.80	0.10	0.03	...	0.99	1.81	0.22	...	...	...	...		7.5
	...	0.92	...	0.18	3.84	...	...	...	1.16	1.98	...	...	...	...	...	8	7.1
Glutamic acid	1.04	...	1.97	1.11	...	...	...	...	0.14	1.17	0.98	...	2.08	...	...		8.6
	1.00	...	2.03	1.16	...	...	...	...	0.07	1.12	1.16	...	2.06	...	...	8	8.2
Proline	...	...	...	1.03	...	...	...	...	...	...	0.87	...	0.91	...	...		2.9
	...	...	...	0.82	...	...	...	...	...	...	0.90	...	0.94	...	...	3	3.3
Glycine	0.07	2.00	2.94	...	2.23	...	1.02	...	1.12	1.19	...	3.10	...	1.09	...		14.0
	...	2.24	3.02	...	2.00	...	1.03	...	1.11	1.10	0.18	3.12	...	1.17	...	14	13.8
Alanine	1.05	2.00	2.15	...	3.05	...	1.00	...	1.23	1.19	...	...	4.07	5.15	...		20.9
	1.04	2.21	2.20	...	3.00	...	0.95	...	1.20	1.04	...	...	4.04	5.20	...	20	19.7
Cysteine‡	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...		0.8
	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	1	1.0
β-Aminoethyl-cysteine	...	...	...	...	...	...	...	...	...	0.83	...	...	...	...	...		...
	...	...	...	...	...	...	...	...	...	1.00	...	...	...	...	...		...
Valine§	0.87	1.01	1.77	1.90	...	0.94	...	...	0.88	...	1.01	1.91	...	2.82	...		13.9
	0.84	1.05	1.95	2.04	...	1.11	...	...	0.86	...	0.94	1.83	...	2.90	...	14	13.4
Methionine	...	...	...	...	0.76	...	...	...	...	...	...	0.81	...	...	...		1.9
	...	...	...	...	1.00	...	...	...	...	...	...	0.90	...	...	...	2	1.8
Isoleucine	...	...	...	...	0.80	...	...	...	0.88	...	...	1.75	...	...	...		3.8
	...	...	...	...	0.92	...	...	...	0.84	...	...	1.78	...	...	...	4	4.1
Leucine	1.08	1.01	1.00	2.03	1.12	...	...	...	2.88	2.02	1.04	4.25	...	0.93	...		16.3
	1.02	0.98	1.00	1.90	0.96	...	...	...	3.00	1.85	1.02	4.13	...	1.12	...	17	17.5
Tyrosine	...	...	...	0.94	0.79	...	...	...	...	...	...	...	...	...	1.00		2.9
	...	...	...	0.87	0.88	...	...	...	...	...	...	...	...	...	1.00	3	2.5
Phenylalanine	...	...	...	...	1.74	...	...	...	1.14	0.92	0.94	...	1.94	...	...		6.9
	...	...	...	...	2.00	...	...	...	0.89	0.88	1.00	...	2.06	0.17	...	7	7.2
Tryptophan	...	1.00	...	1.00	...	...	...	...	...	...	...	...	...	...	...		2.0
	...	1.00	...	1.00	...	...	...	...	...	...	...	...	...	...	...	2	2.0
No. of residues	8	9	13	10	19	2	4	1	16	13	9	16	12	12	2	146	146
Yield %	65	56	43	77	75	59	66	71	57	69	43	69	77	82	100		

* The upper figure in each group refers to SEC, while the lower figure refers to C57BL/6 hemoglobin.[7, 8] † All values for serine and threonine are extrapolated to zero time.
‡ Determined as cysteic acid. § All values for valine greater than one residue were determined by 72-hr hydrolysis.

TABLE 2

AMINO ACID COMPOSITIONS OF THE TRYPTIC PEPTIDES FROM C57BL/6 AND SEC α CHAINS

Amino acid	Amino Acid Residues per Molecule of Peptide														Sum of residues of tryptic peptides	Residues per α chain
	αTp-I	αTp-II	αTp-III	αTp-IV	αTp-V	αTp-VI	αTp-VII	αTp-VIII	αTp-IX	αTp-X	αTp-XI	αTp-XII	αTp-XIII	αTp-XIV		
Lysine	1.00*	1.00	1.00	...	1.00	0.98	1.07	1.00	1.02	...	1.00	1.06	1.02	...	11	11.1
	1.00	0.96	1.04	...	1.08	0.91	0.94	1.00	0.96	...	0.94	1	1.22	...	11	11.1
Histidine	...	...	...	1.07	...	2.06	0.94	...	2.90	...	...	3.80	...	...	11	10.6
	...	...	...	0.97	...	1.90	1.11	...	2.77	...	...	4	0.23	...	11	10.8
Arginine	...	...	...	1.00	...	...	...	...	...	1.00	...	...	...	1.05	3	3.0
	...	...	...	1.03	...	...	...	...	...	1.03	...	...	...	1.04	3	3.1
Aspartic acid	1.12	1.04	...	...	...	1.01	...	...	4.10	...	2.00	2.04	...	...	11	11.7
	1.00	1.09	...	...	...	1.02	...	...	4.88	...	1.83	2	1.31	...	13	12.5
Threonine†	...	...	...	...	1.89	0.97	...	...	0.55	...	...	1.85	2.08	...	7.5	6.9
	...	...	...	...	1.83	0.63	...	...	...	...	...	2	1.18	...	6	6.6
Serine†	1.10	0.91	...	...	0.95	1.94	...	...	2.55	...	...	2.80	2.80	...	13.5	13.0
	0.95	0.85	...	...	0.95	2.11	...	...	1.86	...	...	3	2.83	...	13	13.4
Glutamic acid	1.01	0.15	...	3.10	...	1.00	...	...	...	...	...	...	...	...	5	5.5
	1.02	...	...	2.70	...	1.08	...	...	...	...	...	...	0.20	...	5	5.4
Proline	...	...	...	...	0.97	1.07	...	...	0.96	...	1.00	2.14	...	...	6	5.6
	...	...	...	...	1.09	0.97	...	...	1.05	...	0.98	2	0.33	...	6	6.0
Glycine	1.07	0.19	1.08	3.89	0.09	1.08	2.00	...	1.99	...	...	...	...	...	11	10.9
	0.99	...	1.03	4.07	...	1.33	1.95	...	2.04	...	...	...	0.37	...	11	11.4
Alanine	...	0.21	1.92	2.96	1.10	1.04	...	...	8.06	...	...	3.84	1.20	...	20	19.6
	...	...	1.94	3.25	0.95	1.06	...	...	8.30	...	...	4	1.12	...	20	20.3
Cysteine‡	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0.9
	...	...	...	...	...	...	...	...	...	...	...	1	...	...	1	0.8
β-Aminoethyl-cysteine	...	...	...	...	...	...	...	...	...	...	...	1.00	...	...	...	...
Valine§	0.95	...	...	...	...	1.86	...	...	0.93	...	1.93	2.21	2.14	...	10	10.2
	0.99	...	...	...	...	1.99	...	...	1.01	...	2.23	2	1.93	...	10	10.0
Methionine	...	...	...	...	0.79	...	...	...	...	...	...	...	...	...	1	0.8
	...	...	...	...	0.75	...	...	...	...	...	...	...	...	...	1	0.8
Isoleucine	...	0.88	...	0.93	...	...	...	...	...	...	...	...	...	...	2	2.1
	...	0.95	...	0.94	...	...	...	...	...	...	...	...	0.15	...	2	2.0
Leucine	1.00	0.13	...	0.97	...	...	...	...	6.25	1.00	...	5.80	2.18	...	17	16.7
	0.99	...	...	0.97	...	...	...	...	6.18	0.97	...	6	1.97	...	17	16.5
Tyrosine	...	...	...	0.89	...	0.97	...	...	...	...	...	...	...	0.95	3	2.6
	...	...	...	1.07	...	0.90	...	...	...	...	...	...	...	0.96	3	3.0
Phenylalanine	...	...	...	...	1.92	2.00	...	...	...	...	1.16	1.08	0.82	...	7	7.0
	...	...	...	...	1.96	1.90	...	...	...	...	1.02	1	1.02	...	7	6.8
Tryptophan	...	...	1.00	...	...	...	...	...	...	...	...	...	...	...	1	1.0
	...	...	1.00	...	...	...	...	...	...	...	...	...	...	...	1	1.0
No. of residues	7	4	5	15	9	16	4	1	29	2	7	28	12	2	141	141
Yield %	87	110	57	72	38	77	64	77	85	89	49	45	34	100		

* The upper figure in each group refers to SEC, while the lower figure refers to C57BL/6 hemoglobin.[7,8] † All values for serine and threonine are extrapolated to zero time.
‡ Determined as cysteic acid. § All values for valine greater than one residue were determined by 72-hr hydrolysis.

αTp-IX was compared with C57BL/6 peptide, αTp-IX had one less asparagine which was replaced by half a residue of serine and half a residue of threonine (Table 2).

To decide whether the observed values for serine and threonine were the result of contamination, this peptide was repurified on Dowex 1-X2, but the amino acid composition remained unchanged (Table 3). In addition, we were able to isolate the peptide αTp-VIII-IX, where αTp-VIII is lysine. Because of this additional lysine residue, αTp-VIII-IX was eluted later than αTp-IX and would not be expected to have the same contaminant. Upon amino acids analysis, 0.5 residues of threonine and 2.5 residues of serine were still present (Table 3); therefore, contamination seemed most unlikely. One stage of the Edman degradation removed all of the valine (Table 3), thus eliminating the possibility that the peptide was twice its presumed molecular weight. Since the values for serine and threonine were those found after extrapolation to zero hydrolysis time,[14] the nonintegral values of serine and threonine cannot be explained by partial destruction during acid hydrolysis.

One explanation for these results could be that αTp-IX is a mixture of two peptides which differ by serine or threonine at the same position and which even our most selective separation techniques have failed to resolve. If this were indeed the case, subtractive Edman degradations should remove half residues of both serine and threonine at the same step. To facilitate the interpretation of the results of the Edman degradations, αTp-IX was digested with pepsin to obtain a smaller peptide containing the residues in question. A smaller peptide αTp-IX Pep 5 was isolated by chromatography on Dowex 50-X2 as shown in Figure 2. The material in the crosshatched area contained nonintegral values of serine and threonine (Table 4). This peptide was repurified on Dowex 1-X2 and was submitted to subtractive Edman degradation. The first step removed alanine (Table 4). After the second step, amino acid analysis revealed that both half residues of serine and threonine had practically disappeared (Table 4). Because of the loss of both serine and threonine residues at the same step, it is clear that they both occupy the same position in the sequence but in two different peptides. From the composition and partial sequence of αTp-IX in SEC and C57BL/6, we have been able to assign serine and threonine to positions 68 in the α chains.

Discussion.—From the amino acid compositions of the α- and β-chain tryptic

TABLE 3

AMINO ACID COMPOSITIONS OF TRYPTIC PEPTIDES CONTAINING THE DOUBLE REPLACEMENT

	Residues per Molecule of Peptide			
Amino acid	αTp-IX	Repurified αTp-IX	αTp-VIII–IX	Edman degradation of αTp-IX, stage 1
Lysine	1.00	0.94	1.85	1.01
Histidine	2.90	3.10	3.19	3.06
Aspartic acid	4.10	3.97	4.22	4.00
Threonine*	0.55	0.52	0.55	0.46
Serine*	2.55	2.61	2.65	2.63
Proline	0.96	0.88	1.05	1.04
Glycine	1.99	2.06	2.24	2.22
Alanine	8.06	8.15	8.12	8.10
Valine†	0.93	1.04	1.15	0.09
Leucine	6.15	5.80	5.98	6.19

* All values for serine and threonine are extrapolated to zero time.
† All values for valine greater than one residue were determined by 72-hr hydrolysis.

peptides from SEC and C57BL/6 hemoglobin,[7, 8] it appears that they differ only in the α chain. While we cannot account for the previously reported differences in the β-chain fingerprints,[2] the lack of any distinguishing differences in the α-chain fingerprints can be easily rationalized since the substitutions found do not involve a change of charge.

It is evident from the sequence analysis of αTp-IX that SEC mice actually possess two types of α chains. One type has a serine at position 68, while the other has a threonine residue at this location. In C57BL/6 hemoglobin only one α chain is present which contains asparagine at position 68.

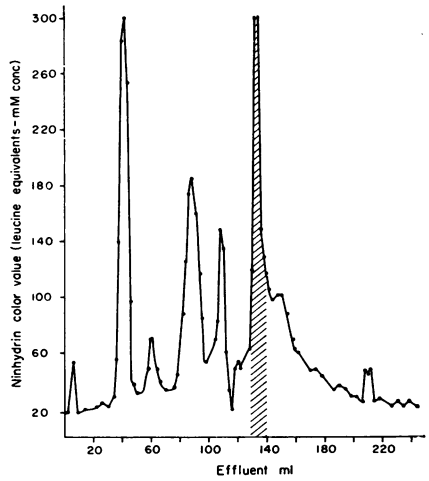

FIG. 2.—Fractionation of the peptic hydrolysate of αTp-IX on Dowex 50 × 2 using a linear gradient of pyridine formate buffers. The cross-hatched area contained the peptic peptide αTp-IX Pep 5 which had the nonintegral values of serine and threonine.

Several hypotheses can be advanced to account for our results. One of these is that we are dealing with hemoglobins from a heterozygous population. Two other examples of double substitutions at the same site have been reported for human heart cytochrome c[15] and bovine carboxypeptidase A.[16] These results have been explained as arising from heterozygosity in the population sample selected for analysis. Although this may indeed be the case for the human and bovine proteins, this explanation is most unlikely in the case of the mouse hemoglobins because of the mating history of this strain of mice (inbred for 82 generations).[17]

A second possibility is that the two SEC α chains could have arisen as the result of a duplication of the α-chain gene followed by a mutation in one of the two genes at the site specifying the amino acid at position 68α. The scheme of gene duplication followed by mutation and translocation had been suggested previously by Ingram[18] to account for the evolution of the four different types of human hemoglobin chains from a common precursor. This hypothesis is extremely difficult to prove, but it cannot be ruled out.

Another explanation is that SEC hemoglobin could be a result of *in vivo* ambiguity.

TABLE 4

SUBTRACTIVE EDMAN DEGRADATIONS OF THE PEPTIC PEPTIDE αTp-IX PEP 5

	Residues per Molecule of Peptide		
Amino acid	αTp-IX Pep 5	Edman degradation of αTp-IX Pep 5, stage 1	Edman degradation of αTp-IX Pep 5, stage 2
Histidine	1.00	0.92	1.03
Aspartic acid	0.02	0.03	0.04
Threonine*	0.40	0.37	0.09
Serine*	0.62	0.59	0.14
Glutamic acid	0.02	0.02	0.07
Glycine	1.04	1.03	1.12
Alanine	2.92	2.04	1.95
Leucine	1.02	0.96	0.93

* All values for serine and threonine are extrapolated to zero time.

This involves the assumption that the nucleotide triplet coding for residue 68 in SEC α chains is a triplet not frequently used and that it can be recognized both as a serine or threonine codon by the tRNA's responsible for the incorporation of these amino acids. That a particular triplet can code for more than one amino acid has been shown in the case of the suppression of a nonsense triplet in alkaline phosphatase from *E. coli*.[19] Double substitutions at the same site also have been found in rabbit hemoglobin[20, 21] and f_2 coliphage coat protein.[22] These results can be accounted for by ambiguity since the genetic history of these sources makes heterozygosity an unlikely possibility.

Summary.—The α and β chains of SEC hemoglobin have been examined and have been found to differ from C57BL/6 hemoglobin at two positions in the α chain. At one of these positions a single replacement occurred, while at the other location, residue 68, two amino acids were found which required that two types of α chains be present. The possible sources of two different α chains in a homozygous strain are discussed.

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