Leghemoglobin: Sequence of Amino Acids around the two Histidyl Residues — A Correction

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The sequence of amino acids around the two histidyl residues of soybean leghemoglobin has recently been described by Ellfolk and Sievers.[1] We wish to report that we have also determined these sequences and have obtained almost but not quite identical results.

The crude leghemoglobin, from soybean plants (*Glycine max* Merr. var. Merrit or Chippewa) infected with *Rhizobium japonicum*, was prepared by Klucas and Russell according to the anaerobic method described by Koch *et al.*[2] The pink supernatant solution was fractionated with ammonium sulfate; the leghemoglobin was in the fraction precipitating between 40 and 85 %. This cut was suspended in 0.1 M potassium phosphate buffer, of pH 7.0, frozen and shipped to us in dry ice. The leghemoglobin was converted to the CN-Met form by treatment with excess $K_3Fe(CN)_6$ and KCN. The material was dialyzed against 0.1 M phosphate buffer, pH 7.5, and chromatographed on a column of Sephadex G-100. The leghemoglobin fraction was concentrated by pressure filtration in a Diaflo cell with a UM-1 membrane. The components (I, II, III) of this fraction were isolated by chromatography on DEAE-Sephadex at pH 7.7 with a linear gradient between 0.05 M tris-HCl and the same buffer containing 0.2 M NaCl. Globin (100 mg), prepared by treatment of component I (Ellfolk's "slow" component) with 1.5 % acid acetone at $-13°C$,[3] was suspended in 10 ml of 0.5 % $(NH_4)HCO_3$, heat denatured at 90° for 10 min and digested with trypsin (TPCK, Worthington) in a 1:50 weight ratio for 3 h at room temperature. Tryptic peptides were separated by high voltage electrophoresis on Whatmann 3MM paper at pH 6.0. The amino acid compositions of the histidyl peptides (T-1, T-2) are given in Table 1. The sequence of the 7 residue peptide, T-1, determined directly by a

Table 1. The amino acid composition of the histidyl tryptic peptides from apoleghemoglobin-1 and the peptic peptides derived from T-2.

	T-1	T-2	P-1	P-2	P-3	P-4	P-5	P-6
Lys	1.00	0.98		1.00		0.86	1.00	
His	0.98	0.92		0.64		0.71	0.83	
Asp		1.13	0.07	0.91	1.03			
Thr	0.98	0.96	0.85	0.18	0.87			
Ser		2.02	1.00	1.00	0.90	0.95	0.92	
Glu	1.02	1.12	0.11	1.18	0.09	0.90	1.13	
Gly	1.05	2.14	1.04	1.18	1.10	1.10	1.08	
Ala	1.02	4.64	1.70	2.18 [a]	2.03	2.33 [a]	1.25	1.95
Val		2.30 [a]	1.33 [b]	0.45	2.16	0.62	0.79	
Leu	0.93	1.04		0.82		0.57		1.00
Yield-%	63	30	10	10	40	30	40	8
Number of residues	7	18	7	11	8	10	7	3

Peptides are designated T for tryptic and P for peptic. Analyses were obtained after 24 h hydrolysis in sealed, evacuated tubes at 110° in 6 N HCl.

[a] Found to be 3 residues by sequence determination.

[b] Found to be 2 residues by sequence analysis.

The yield of peptide was calculated from the micromoles of the apoprotein or the peptide digested.

Histidyl sequence I

																		Mutation distance[7]
LHb	-Ala	Ser	Gly	Thr	Val	Val	Ala	Asp	Ala	Ala	Leu	Gly	Ser	Val	His	Ala	Glx Lys-	
β-Human[6]	-Leu	Asp	Asn	Leu	Lys	Gly	Thr	Phe	Ala	Thr	Leu	Ser	Glu	Leu	His	Cys	Asp Lys-	
	78									88				92				
Base changes	2	2	2	2	2	1	1	2	0	1	0	2	2	1	0	2	1 0	23
α-Human	Val	Asp	Asp	Met	Pro	Asn	Ala	Leu	Ser	Ala	Leu	Ser	Asp	Leu	His	Ala	His Lys	
Base changes	1	2	1	1	2	2	0	2	1	0	0	2	2	1	0	0	1 0	18
Lampetra[8]	Asp	Asp	Thr	Glu	Lys	Met	Ser	Met	Lys	Asp	Leu	Ser	Gly	Lys	His	Ala	Lys	
	89																105	
Base changes	1	2	2	2	2	1	1	2	2	1	0	2	2	2	0	0	1	23

Histidyl sequence II

								Mutation distance[7]
LHb I	-Leu	Thr	Gly	His	Ala	Glx	Lys-	
β-Human	-Val	Lys	Ala	His	Gly	Lys	Lys-	
Base changes	1	1	1	0	1	1	0[66]	5
α-Human	-Val	Lys	Gly	His	Gly	Lys	Lys	
Base changes	1	1	0	0	1	1	0	4

Table 2. Summary of sequence data on the histidyl peptides from leghemoglobin-I and comparison with human and lamprey hemoglobin sequences containing the proximal and distal histidines. Arrows represent successful steps of the Edman degradation; prefixes T, P, and N refer to tryptic, peptic, and pronase digestions, respectively.

multiple subtractive Edman degradation technique [4] with amino acid analysis after removal of each residue, confirmed the results of Ellfolk and Sievers;[1] our procedure, however, did not distinguish between glutamic acid and glutamine.

The sequence of T-2 was derived as follows. Five steps of the Edman degradation were carried out on 0.35 μmoles of T-2; the peptide remaining was digested with pronase (1:1000) at room temperature. The resulting peptides were separated by high voltage electrophoresis. One tripeptide and one tetrapeptide were sequenced by the Edman procedure. Another 0.2 μmoles of T-2 were digested with pepsin (Worthington, 0.125 mg pepsin/μmole peptide) in 5 % formic acid at room temperature for 4 h. The pepsin was added in two equal aliquots, at zero time and at 2 h. The amino acid composition of each peptide isolated by electrophoresis is given in Table 1; the basis for the sequence is summarized in

Table 2. The low yield of Val in T-2 and P-1 is expected since Val-Val bonds are not completely hydrolyzed in 24 h.[5] Table 2 compares the sequence of the relevant parts of the α and β chains of human hemoglobin and the hemoglobin of the lamprey with these histidyl peptides from leghemoglobin-1.

These results for T-2 differ from those reported by Ellfolk and Sievers only in positions 5 — 7. Our data indicate this sequence to be Val-Val-Ala whereas Ellfolk and Sievers report it to be Val-Ala-Val. We believe that our direct Edman degradation is conclusive. Their peptide Pa-5 (in their Tables 2 and 3) supposedly has only a single Val, *yet* their amino acid analyses indicate 1.8, 1.6 and 1.6 valyl residues after steps 1, 2, and 3, respectively. We do not think the differences in amino acid composition of Pa-3 and Pa-5 reported in their Table 3 are significant: 1.51 Val and 1.21 Val were found in Pa-3 and Pa-5, respec-

tively. Val-Val bonds are extremely resistant to hydrolysis and low yields would be anticipated after hydrolysis in 6 N HCl at 108° for only 18 h. Their results with carboxypeptidase A digestion of Pa-5 do not really distinguish between a Val-Val-Ala and a Val-Ala-Val sequence. In any event, our finding of a tripeptide N-1 (Table 2) which has the sequence Val-Ala-Asp would appear to be conclusive.

Several structural features of the leghemoglobin-1 sequences are common to those of animal hemoglobins. In particular, Leu and His (residues 88 and 92 in the human β chain) appear invariant. This leucine makes an important contact with the heme and His 92 is linked to the iron atom. Comparison of the sequences in terms of base changes is given in Table 2. Leghemoglobin-1 may be homologous with animal hemoglobins; both may have arisen from a common ancestor. The sequences also suggest that the heme in leghemoglobin may be partially buried in a hydrophobic pocket as it is in animal hemoglobins.

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Djurleite Synthesis in Low Temperature Aqueous Solution

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A synthetic phase, corresponding to the mineral djurleite,[1,2] $Cu_{1.97}S$, has been identified as the major product of the reaction between cuprous oxide and aqueous sodium sulphide solution at 25°C and 1 atm pressure.

100 mg analytical grade Cu_2O, suspended in 10 ml oxygen-free deionized water, was placed in a 500 ml Kilner jar. 23.4 ml analytical grade Na_2S solution (100 g/l) was made up to 300 ml with O_2-free deionized water and 10 % analytical grade HCl to the required pH. The poised sulphide solutions were added to the Cu_2O suspension, and the Kilner jar sealed under nitrogen. 10 runs with Na_2S solutions of pH = 6.81 to 8.91 ($E_h = -129$ to -192 mV) were made.

The precipitates were aged for 14 days at 25°C with intermittent agitation. The pH and E_h of the solutions were measured. The pH values found varied from 7.03 to 10.71 ($E_h = -185$ to -247 mV). Samples of the precipitates were sealed in 0.3 mm bore Lindemann glass capillaries and analysed, wet, by X-ray powder diffraction analysis in an 11 cm Debye-Scherrer camera with $CuK\alpha$ radiation. The remainder of the products were filtered through sintered glass filters, washed in O_2-free deionized water and alcohol, and dried at room temperature in a vacuum desiccator. X-Ray powder diffraction photographs were obtained from a Guinier focussing camera with $CuK\alpha_1$ radiation and a KCl internal standard.

X-Ray data for the products of run C201 are shown in Table 1. In this run the pH of the sodium sulphide solution was 7.96 ($E_h = -136$) and the final pH of the reaction mixture 10.30 ($E_h = -162$). The products were identified as major djurleite admixed with minor covellite. The djurleite reflections were extracted and indexed by desk calculation according to an orthorhombic cell[3] with cell dimensions of $a = 26.9$, $b = 15.5$, $c = 13.3$ Å. Further mathematical refinements were not attempted with these data because the reflections were too diffuse to give greater