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Synthesis of a Protected Sperm Whale Myoglobin-(77—96)-Eicosapeptide and Circular Dichroism Spectra of the Related Peptides

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A protected sperm whale myoglobin-(77—96)-eicosapeptide (**23**) was synthesized by a solution method. The protected peptide **23** could be effectively purified by silica-gel column chromatography with 1-butanol-acetic acid-water as the eluate. The CD spectra of protected fragment peptides were measured in a solution of 2,2,2-trifluoroethanol. A protected sperm whale myoglobin-(85—96)-dodecapeptide and **23** showed CD profiles characteristic of a helical structure.

Myoglobin is a globular heme protein which plays a role in the storage of oxygen in the cells of muscle. The myoglobin molecule of higher animals is composed of a globin chain containing 153 amino acid residues (mol wt 17000) and a protoheme. A higher-order structure of sperm whale myoglobin was elucidated by means of an X-ray analysis by Kendrew et al.[1)] The E and F regions of sperm whale myoglobin include distal (E7) and proximal (F8) histidine residues which coordinate a heme iron atom, and are important sites for producing physiological functions. It is of interest to synthesize a peptide of the E-EF-F region and to investigate the structure and functional properties of the peptide in comparison with those of myoglobin. However, few studies have been carried out on the synthesis of those regions of the myoglobin. In the past Eckstein and Bayer[2)] tried to synthesize the sperm whale myoglobin-(81—94)-peptide using two methods involving fragment condensation: between positions 81—82 and 83—94, and between positions 81—88 and 89—94. However, they could not obtain the desired peptide.

As an initial approach regarding the subject mentioned above, we synthesized a protected sperm whale myoglobin-(77—96)-eicosapeptide (**23**). Peptide **23** consists of EF and F segments of the myoglobin and includes the important proximal histidine residue. The synthesis of **23** carried out by a solution method which makes it possible to obtain some protected intermediate peptides of various size. Various problems in the synthesis are described in "Results and Discussion". The CD spectra of **23** and the fragment peptides were measured in order to investigate whether those protected peptides can form a helical structure. The helical property deduced from the CD spectra of each peptide was correlated with the secondary structure, shown by X-ray analysis,[1)] of the corresponding part of myoglobin.

Results and Discussion

The protected eicosapeptide **23** was built up from four fragment peptides by the route illustrated in Fig. 6. These four fragment peptides were prepared by a stepwise elongation method, according to the routes showed in Figs. 1—5.

Peptide in Positions 91—96. Figure 1 illustrates the synthesis of fragment (**5**), Boc-Gln-Ser(Bzl)-His-Ala-Thr-Lys(Z)-OBzl.[3)] Boc-His(Boc)-Ala-Thr-Lys(Z)-OBzl (**3**) was constructed stepwise with Boc-Thr-OH, Boc-Ala-OH, and Boc-His(Boc)-OH using H-Lys(Z)-OBzl as starting material by a mixed anhydride (MA) method.[4)] Boc-Thr-Lys(Z)-OBzl (**1**) was purified by chromatography on a silica-gel column with CH_2Cl_2-MeOH systems as eluates. Compound **3** was contaminated with a small amount of impurities, but used for the following coupling without further purification. After the treatment of **3** with trifluoroacetic acid (TFA), the tetrapeptide ester was condensed with Boc-Ser(Bzl)-OH using EDC[5)] in the presence of HOBt[6)] to give Boc-Ser(Bzl)-His-Ala-Thr-Lys(Z)-OBzl (**4**). The pentapeptide ester derived from **4** was coupled with Boc-Gln-ONp by an active ester method.[7)] Product **5** was purified into a chromatographyically homogeneous form by gel filtration on Sephadex LH-20.[8)]

Peptide in Positions 85—90. Figure 2 shows the synthesis of fragment (**11**), Boc-Glu(OBzl)-Leu-

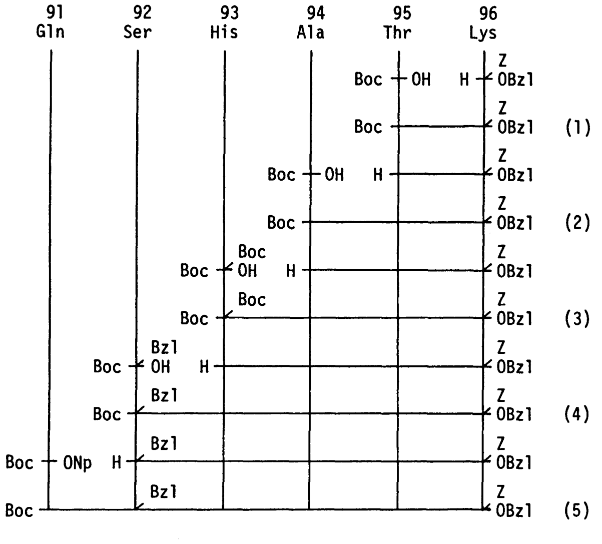

Fig. 1. Synthetic scheme of positions 91—96.

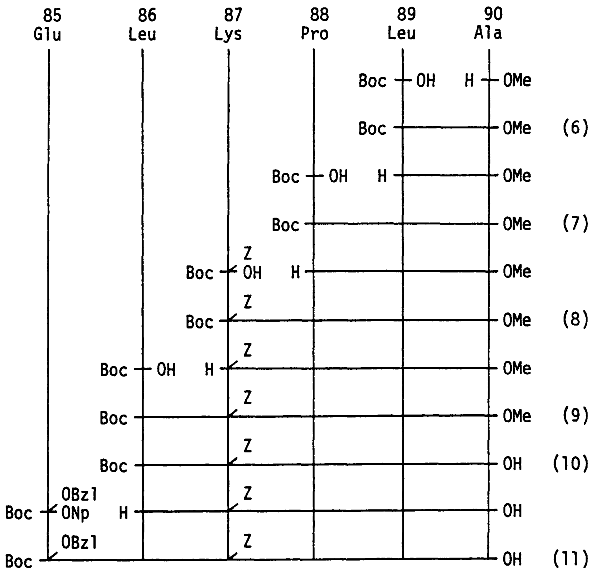

Fig. 2. Synthetic scheme of positions 85—90.

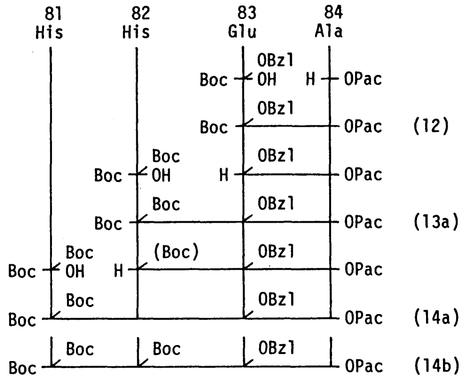

Fig. 3. Synthetic scheme of positions 81—84.

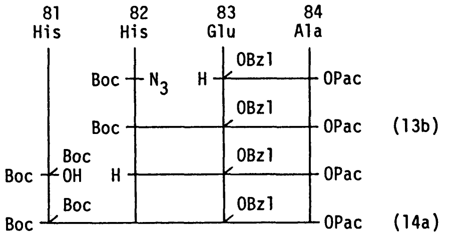

Fig. 4. Alternative synthetic scheme of positions 81—84.

Lys(Z)-Pro-Leu-Ala-OH. In the synthesis of Boc-Leu-Lys(Z)-Pro-Leu-Ala-OMe (**9**), the starting material, H-Ala-OMe, was elongated stepwise with Boc-Leu-OH, Boc-Pro-OH, Boc-Lys(Z)-OH, and Boc-Leu-OH by means of the MA or EDC-HOBt method. Compound **9** was purified by the use of a Sephadex LH-20 column. The methyl ester **9** was hydrolyzed to give Boc-Leu-Lys(Z)-Pro-Leu-Ala-OH (**10**). After removing the N-terminal protecting group of **10**, the resulting partially protected pentapeptide, TFA·H-Leu-Lys(Z)-Pro-Leu-Ala-OH, was neutralized and, then, coupled with Boc-Glu(OBzl)-ONp by an active ester method to afford **11**. Fragment **11** was purified by gel filtration.

Peptide in Positions 81—84. Figures 3 and 4 illustrate the routes employed in the preparation of fragment (**14a**), Boc-His(Boc)-His-Glu(OBzl)-Ala-Opac. The coupling of H-Ala-Opac with Boc-Glu(OBzl)-OH proceeded in yields 43—56% by the MA method, and in an yield of 74% by the EDC-HOBt procedure. Compound **12** was treated with TFA and the dipeptide ester was condensed with Boc-His(Boc)-OH by the MA method to give Boc-His(Boc)-Glu(OBzl)-Ala-Opac (**13a**). After the treatment of **13a** by TFA for 70—340 min at room temperature, the product showed two spots on TLC. The treatment of **13a** with 4 M[†] HCl/dioxane for 3 h at room temperature also gave a similar result. The two components seemed to be peptides in which, respectively, one and two Boc groups were removed with an acid-treatment. Therefore, the mixture containing the two components was coupled without purification with Boc-His(Boc)-OH by the EDC-HOBt method to yield **14a**. The crude product, including two components (A and B), was chromatographed on a silica-gel column and the two components were separately isolated. Component A was identified as Boc-His(Boc)-His(Boc)-Glu(OBzl)-Ala-Opac (**14b**), and component B as the desired compound **14a** by elemental and FAB mass analyses. Fragment **14a** was also synthesized by another route, illustrated in Fig. 4. The Boc-His-N_3 derived from corresponding hydrazide was coupled with the H-Glu(OBzl)-Ala-Opac to prepare Boc-His-Glu(OBzl)-Ala-Opac (**13b**) by the Honzl and Rudinger method.9) After the deprotection of **13b** with TFA, the tripeptide ester was condensed with Boc-His(Boc)-OH by the MA method to yield **14a**. Analytical data regarding component B agreed with that of **14a** synthesized by the route shown in Fig. 4.

Peptide in Positions 77—80. Figure 5 outlines the synthesis of fragment (**18**), Boc-Lys(Z)-Lys(Z)-Lys(Z)-Gly-OH. The starting material, H-Gly-OEt, was succesively elongated by Boc-Lys(Z)-OH with the MA method to produce fragment (**17**), Boc-Lys(Z)-Lys(Z)-Lys(Z)-Gly-OEt. Product **17** was converted to the corresponding acid **18** by saponification. The synthesis of **18** proceeded smoothly and requires no additional comments.

Peptide in Positions 77—84. The removal of the Boc group of **14a** was carried out smoothly by treating with TFA under the usual conditions. The resulting peptide gave a single spot on TLC and was confirmed to be 3TFA·H-His-His-Glu(OBzl)-Ala-Opac (**19**) by elemental analysis. The tetrapeptide ester **19** was coupled with **18** in the presence of HOBt by EDC to yield protected octapeptide **20**. The product **20** was reduced over zinc powder in acetic acid10) to afford fragment (**21**), Boc-Lys(Z)-Lys(Z)-Lys(Z)-Gly-His-

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[†] 1 M=1 mol dm^{-3}.

His-Glu(OBzl)-Ala-OH.

Peptide in Positions 85—96. Several methods were investigated for the synthesis of fragments (positions 85—96 or 87—96). Acyl components, Boc-Glu(OBu^t)-Leu-Lys(Z)-Pro-Leu-Ala-N_3 and Boc-Lys(Z)-Pro-Leu-Ala-N_3, respectively, were condensed with an amino component, H-Gln-Ser-His-Ala-Thr-Lys(Z)-OH. The yields of both couplings were very low. Also, the coupling of the former azide with an amino component, H-Gln-Ser-His-Ala-Thr-Lys(Z)-OBzl, did not give a satisfactory result. The low yield in these coupling reactions seems to be attributed to a conversion of the N-terminal residue to pyroglutamyl, since the glutamine residue of a free hexapeptide, H-Gln-Ser-His-Ala-Thr-Lys-OH, easily changed to the pyroglutamyl residue. This reaction was catalyzed by the imidazole ring of the neighboring histidine residue.[11] Therefore, the hydroxyl group of serine was protected by a benzyl group, as one carrying a steric hindrance in order to suppress the effect of the imidazole ring. Also, the coupling method was changed to the EDC-HOBt procedure, a more reactive compared to the azide method. As illustrated in Fig. 6, fragment **5** was treated with TFA and the corresponding hexapeptide ester was coupled with **11** by the EDC-HOBt procedure to prepare fragment (**22**), Boc-Glu(OBzl)-Leu-Lys(Z)-Pro-Leu-Ala-Gln-Ser(Bzl)-His-Ala-Thr-Lys(Z)-OBzl. The yield was 86% and was extremely high compared with those of the three methods described above. The improvement in the yield may be attributed to the use not only of **5**, but also of the reactive EDC-HOBt procedure. Namely, the slower condensation should cause the formation of a greater amount of pyroglutamic acid residue from the N-terminal glutamine residue of the hexapeptide, H-Gln-Ser-His-Ala-Thr-Lys(Z)-[OH or OBzl].

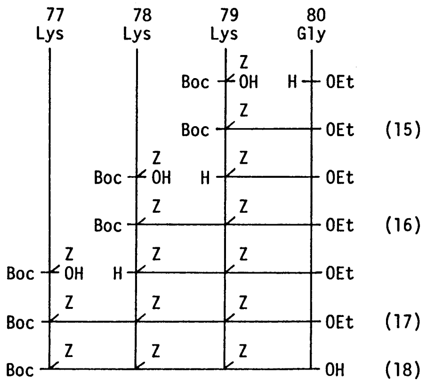

Fig. 5. Synthetic scheme of positions 77—80.

Peptide in Positions 77—96. After removing the Boc group of **22**, the dodecapeptide ester was coupled with **21** in the presence of HOBt by EDC to afford fragment (**23**), Boc-Lys(Z)-Lys(Z)-Lys(Z)-Gly-His-His-Glu(OBzl)-Ala-Glu(OBzl)-Leu-Lys(Z)-Pro-Leu-Ala-Gln-Ser(Bzl)-His-Ala-Thr-Lys(Z)-OBzl. After the purification of **23** by gel filtration, it still contained small amounts of impurities. A part of **23** was further purified by a silica-gel column with 1-butanol-acetic acid-water solvent system as the eluate. The recovery of pure **23** was 24%. The cause of the low recovery is most likely based on adsorption on the silica gel.

As mentioned above, the improvement in fragment condensation between **5** and **11** has enabled the syn-

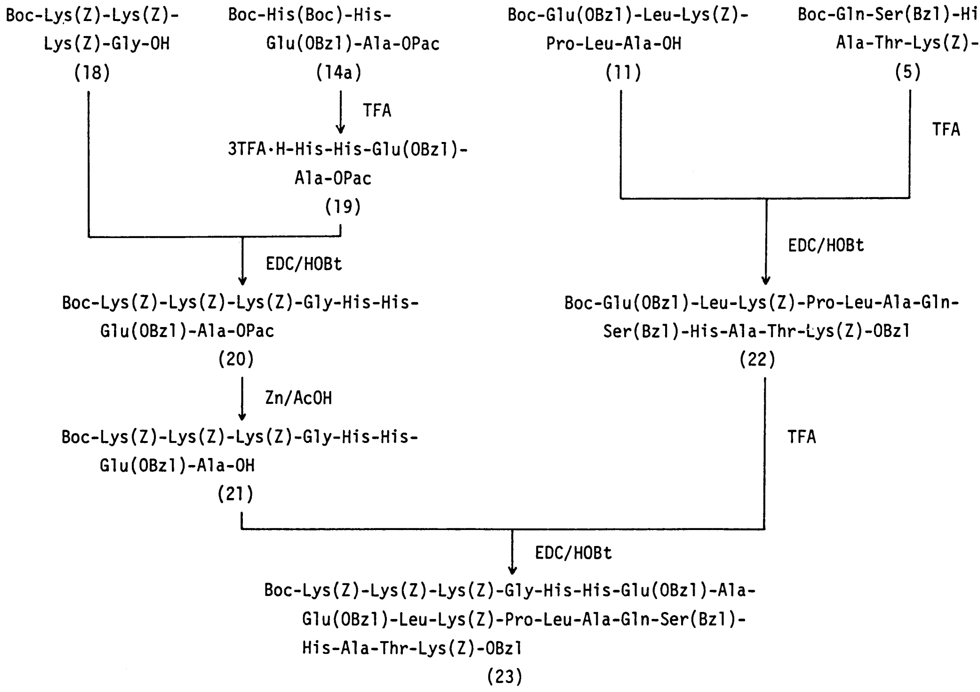

Fig. 6. Synthetic scheme of positions 77—96.

Table 1. Yields, Physical Properties and Analytical Data of Intermediate Protected Peptides[a)]

Compound	Yield/%	Mp θ_m/°C	$[\alpha]_D^{24}$/° (*c* 1.0, MeOH)	Molecular formula	Elemental analysis/% Found (F:), Calcd (C:)			R_f[b)]				
					C	H	N	R_f^1	R_f^2	R_f^5	R_f^6	R_f^7
1[c)]	91	85—90	−19.2	$C_{30}H_{41}O_8N_3$	F: 62.89 C: 63.03	7.39 7.23	7.40 7.35	0.55	0.77			
2	76	115—116	−37.5	$C_{33}H_{46}O_9N_4 \cdot H_2O$	F: 60.29 C: 59.99	7.32 7.32	8.48 8.48	0.38	0.67			
3	74	113—118	−4.2 (DMF)	$C_{44}H_{61}O_{12}N_7 \cdot H_2O$	F: 58.41 C: 58.85	7.16 7.07	10.70 10.92	0.43	0.77			
4	75	141—143	−13.2 (DMF)	$C_{49}H_{64}O_{12}N_8 \cdot H_2O$	F: 60.40 C: 60.36	6.84 6.82	11.48 11.49	0.12 0.02	0.40 0.18	 (trace)		
6[d)]	94	114—114.5	−45.7	$C_{15}H_{28}O_2N_2$	F: 56.72 C: 56.94	9.11 8.92	8.71 8.85	0.83	0.86			0.90
7	95	89—90	−95.6	$C_{20}H_{35}O_6N_3$	F: 57.79 C: 58.09	8.40 8.53	9.99 10.16	0.60	0.83			
8	82	77—79	−78.3	$C_{34}H_{53}O_9N_5$	F: 59.93 C: 60.43	8.00 7.90	10.23 10.36	0.47	0.78			0.86
9	76	79—81.5	−86.1	$C_{40}H_{64}O_{10}N_6 \cdot 1/2H_2O$	F: 60.31 C: 60.21	8.30 8.21	10.60 10.53	0.52	0.72			
12	74	128—129	−38.0	$C_{28}H_{34}O_8N_2$	F: 63.68 C: 63.87	6.59 6.51	5.30 5.32	0.79		0.70		
13a	94	130—131	−25.2	$C_{39}H_{49}O_{11}N_5$	F: 61.21 C: 61.33	6.56 6.47	9.07 9.17	0.49 0.57	0.91 (trace)			
13b	68	79—83	−41.4	$C_{34}H_{41}O_9N_5 \cdot 1/2H_2O$	F: 61.10 C: 60.70	6.53 6.29	10.02 10.41		0.41 0.33	 (trace)		
14a[e)]	44	101—104 (decomp)	−31.8	$C_{45}H_{56}O_{12}N_8 \cdot 1/2H_2O$	F: 59.16 C: 59.40	6.31 6.31	12.21 12.31		0.40			
15[f)]	93	66—68	−12.8	$C_{23}H_{35}O_7N_3$	F: 59.33 C: 59.34	7.80 7.58	9.00 9.03	0.57	0.82	0.42		
16	92	145.5—147	−21.1	$C_{37}H_{53}O_{10}N_5$	F: 60.98 C: 61.06	7.35 7.34	9.53 9.62	0.40	0.73	0.12		
18	99	166—168	−13.4 (DMF)	$C_{49}H_{67}O_{13}N_7$	F: 61.08 C: 61.17	7.11 7.02	10.14 10.19				0.35	0.83

a) The amino acid composition of the acid hydrolyzate of each compound agreed well with that of theoretical values. b) The solvent systems of R_f were showed in experimental section. c) Mp 82—83 °C in lit.[15] d) Mp 113—115 °C in lit.[16] e) This compound was synthesized by the procedure of Fig. 4; FAB mass: *m/z* 901 (MH^+, $C_{45}H_{57}O_{12}N_8$). f) Mp 68 °C in lit.[17]

thesis of the protected eicosapeptide **23**; it will further be helpful in the syntheses of longer peptides. Also, it has been found that a solvent system, 1-butanol-acetic acid-water, well dissolves protected peptides and, accordingly, it is very useful in silica-gel chromatography of protected long peptides.

The purity of all the protected compounds was confirmed by TLC, amino acid analysis of acid hydrolyzate and elemental analysis.

Circular Dichroism Spectra. The CD spectra of protected fragment peptides were recorded in the region of approximately 190 to 270 nm. In the CD measurement the protected fragment peptides and sperm whale myoglobin were dissolved in TFE and in 60% TFE, respectively. The CD data were reported as mean residue ellipticity, $[\theta]$, which were expressed in degree square centimeter per decimole. For calculations of helical contents, the value of $[\theta]_{222}$ for completely helical structure was taken as −32000.[12] The theoretical helicity of a fragment peptide is expressed by the proportion of amino acid residues participating in the helical structure in the known three-dimensional structure of sperm whale myoglobin to the total residues in the fragment peptide. In order to compare the helicity of fragment peptides of different size, the relative helical contents of the fragment peptides were expressed by the helical ratio,[13] which is the ratio of the observed helical content vs. that expected from the position of the fragment peptide in sperm whale myoglobin. In the amino acid sequence of sperm whale myoglobin-(77—96)-peptide, -Lys-Lys-Lys-Gly-His-His-Glu-Ala-Glu-Leu-Lys-Pro-Leu-Ala-Gln-Ser-His-Ala-Thr-Lys-, the underlined amino acid residues take part in the helical structure according to the result of X-ray analysis.

Figure 7 illustrates the CD spectra of four protected fragment peptides in TFE. The spectrum of **11** shows a shoulder at 228 nm and a negative band at 200 nm. The spectra of **20** and **21** exhibits a negative band near 230 nm and a positive band near 210 nm. Fragments **11**, **20**, and **21** do not exhibit the distinguishing CD profiles for a helical structure. The parts corresponding to fragments **20** and **21** scarcely contributed to the helical structure in the sperm whale

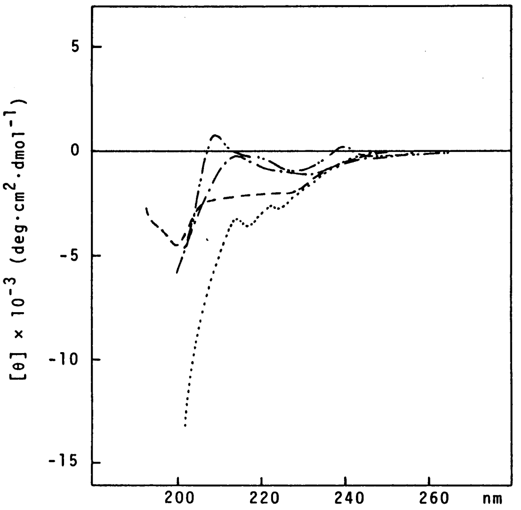

Fig. 7. CD spectra of **5** (·········), **11** (———), **20** (—·—), and **21** (—··—) in TFE.

myoglobin; it is therefore expected that those two peptides do not take a helical structure. Fragment **11** includes a part of the F helical region, though its spectrum did not suggest the exsistence of a helical structure. Fragment **5** also contains a fraction of the F helical region. The spectrum of **5** revealed negative bands at 226 and 217 nm, and suggested the presence of a helical structure, in spite of the small peptide. The helicity of **5** was calculated as 8.4% on the basis of the assumption that its $[\theta]_{222}$ value (−2700) depends only on the helical structure. The theoretical helicity was calculated according to the method stated above. That is, the part corresponding to fragment **5**, within the sperm whale myoglobin, has 4 residues in the helical structure out of 6 residues (theoretical helicity; 67%). Fragment **5**, containing part of a helical segment, is apt to form a helical structure compared with **20** and **21**, including a large portion of a nonhelical segment. However, fragment **11**, containing a helical segment, did not show the presence of a helical structure.

Figure 8 exhibits the CD spectra of two fragments, **22** and **23**, in TFE and sperm whale myoglobin in 60% TFE. The spectra of **22** and **23** showed negative bands at 220 and 205 nm, a cross overpoint at 200 nm and a positive band near 195 nm, and are very similar to that of the myoglobin. These two fragments exhibited conspicuous helical CD profiles. The values of $[\theta]_{222}$ of **22** and **23** were −10400 and −12000, respectively, and the helicity of the two fragments were calculated as being 33% (theoretical helicity; 75%) and 38% (theoretical helicity; 50%), respectively, from those data. It is well-known that TFE used as a solvent stabilizes a helical structure of peptide or protein.

Figure 9 shows the CD spectra of **23** in TFE and TFE containing water. The values of $[\theta]_{222}$ of **23** in TFE, 80% TFE-water, and 60% TFE-water were −12000 (helicity; 38%), −10200 (helicity; 32%), and −8300 (helicity; 26%), respectively. The increment of water content diminished the helical content. These results revealed that the helical structure of **22** and **23** are stabilized in TFE solution. Those two fragments possess hydrophobic protecting groups on the side chains of amino acid residues. The helicities

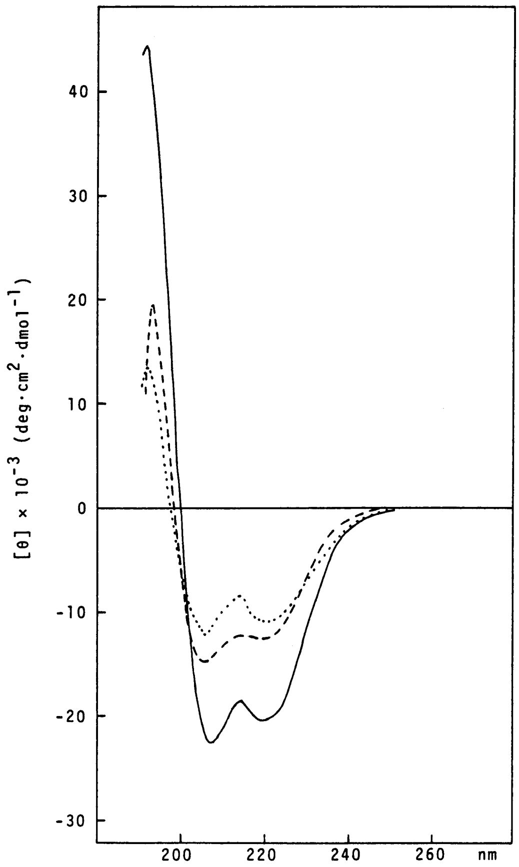

Fig. 8. CD spectra of **22** (·········) and **23** (———) in TFE, and myoglobin (————) in 60% TFE-water.

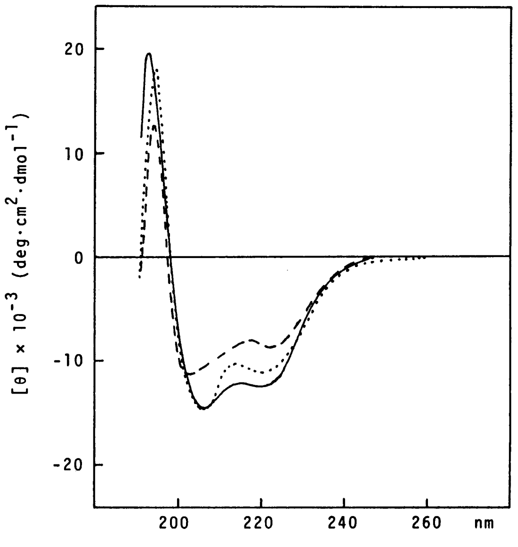

Fig. 9. CD spectra of **23** in TFE (————), 80% TFE-water (·········), and 60% TFE-water (———).

of protecting-group-free peptides derived from **22** and **23** were very small in aqueous solution, and were rather smaller than those of the protected peptides, even in 60% TFE.[14)]

In peptides **22** and **23**, the protection of some residues by hydrophobic groups seems to enhance the helical property of these peptides. The disappearance of the ionic property of the side chains of some residues by protection may also affect the helicity. Fragment **23** was formed by the addition of **21**, including almost nonhelical region to **22**. In spite of the addition of an almost nonhelical segment, the helical content of **23** was somewhat larger than that of **22**. This result implied that the helical structure is stabilized by the interaction among amino acid residues remote in the primary sequence. The helical ratios of **5**, **22**, and **23** were 0.13, 0.44, and 0.76, respectively. The ratios increased with the elongation of the peptide chain. The increase of the helical ratios also suggested the importance of the interaction among remote amino acid residues.

In conclusion, the CD study revealed that fragment peptides **5**, **22**, and **23** have helical structures, even though to various extents. The structures of those compounds seem to be stabilized by the effect of TFE and the hydrophobic interaction among hydrophobic protecting groups and side chains arranged spatially near to each other. This result also suggests that the hydrophobic interaction may be an important factor for the formation of a helical structure in myoglobin. Values of the helical ratio suggest that peptides **22**, and particularly **23**, probably possess a structure similar to the corresponding parts of myoglobin. The suggestion of the existence of a helical structure in peptides **22** and **23** is significant as a prerequisite of the formation of a complex between synthesized peptides and a protoheme in a similar way to that in myoglobin.

Experimental

Melting points were measured by the capillary method and are given as uncorrected values. Optical rotations were determined with a DIP-140 type (Jasco) polarimeter. Amino acid analyses were performed on a JLC-6AS automatic analyzer (JEOL) after acid hydrolysis with 6 M HCl in a sealed tube at 110 °C for 20—21 h. The theoretical values of amino acid ratios are shown in parentheses after each results.

In synthetic procedures, the imidazole functional group of histidine was temporarily protected by the Boc group on the occasion of incorporation of a histidine residue into a peptide chain. The functional groups of side chains, other than the hydroxyl group of threonine, were protected. The N^{α}- and N^{im}-Boc protecting groups were removed by TFA in the presence of anisole, or TFA at room temperature for 45—180 min. After evaporation of the solvents, the residue was triturated with diethyl ether to give a product. The removal of Boc groups of compounds **1**, **2**, **3**, **6**, **7**, **8**, **12**, **13b**, and **15** were carried out by a method similar to this method. Compounds **1**, **2**, **3**, **6**, **7**, **9**, **13a**, **14a** in Fig. 4, **15**, and **16** were synthesized by the MA method, compounds **4**, **8**, and **12** by the EDC-HOBt method and compound **13b** by the azide method. After couplings, the organic layers were washed successively with water, 5% citric acid, water, 5% sodium hydrogencarbonate, and water, and then dried over anhydrous sodium sulfate except when noted otherwise. Compound **18** was prepared from **17** by saponification. The evaporation of solvents was carried out under reduced pressure. Unless stated otherwise, residues and crystals were dried over NaOH pellets and P_2O_5 in vacuo at room temperature. Silica gel 60 (Merck and Co.) was used in column chromatography if not mentioned otherwise.

Thin-layer chromatography was performed on TLC plates silica gel 60 F_{254} and HPTLC plates silica gel 60 F_{254} with a concentration zone, (Merck and Co.), using the following solvent systems (volume ratios): R_f^1, chloroform-methanol (95 : 5); R_f^2, chloroform-methanol (9 : 1); R_f^3, chloroform-methanol (2 : 1); R_f^4, chloroform-methanol (1 : 1); R_f^5, chloroform-ethyl acetate (1 : 1); R_f^6, chloroform-methanol-acetic acid (95 : 5 : 5); R_f^7, chloroform-methanol-acetic acid (9 : 1 : 1); R_f^8, 1-butanol-acetic acid-water (3 : 1 : 1); R_f^9, 1-butanol-acetic acid-water (4 : 1 : 1); R_f^{10}, 1-butanol-pyridine-acetic acid-water (4 : 1 : 1 : 2), R_f^{11}, 1-butanol-pyridine-acetic acid-water (16 : 10 : 3 : 12).

Analytical data of compounds **1**, **2**, **3**, **4**, **6**, **7**, **8**, **9**, **12**, **13a**, **13b**, **14a** in Fig. 4, **15**, **16**, and **18** are given in Table 1.

CD measurements were performed using a J-500 spectropolarimeter with a DP-500N Data Processor. The temperature was kept constant at 24 °C. Cells with light paths of 0.1 and 0.5 mm were used. Measurements of fragment peptides and myoglobin were carried out in solutions at concentrations of 5×10^{-4} M and 5×10^{-5} M, respectively. Sperm whale myoglobin was purchased from Sigma and used without further purification.

Boc-Gln-Ser(Bzl)-His-Ala-Thr-Lys(Z)-OBzl (5). Compound **4** (6.410 g) was treated with TFA (25 ml) and anisole (1.45 ml) in the usual manner and yielded a solid. Boc-Gln-ONp (2.952 g) was dissolved in DMF (10 ml); to this solution was added a solution containing the above-mentioned solid and Et_3N (1.86 ml) in DMF (25 ml). The mixture was stirred for 4.5 d at room temperature and then concentrated. The product was dissolved in MeOH (80 ml), and the solution was applied to a Sephadex LH-20 column (3.6×139 cm). The product was eluted with DMF. Fractions of 10 g each were collected. Fractions 53—77 were combined and the solvent was evaporated. The residue was recrystallized from methanol-diethyl ether; 5.816 g (80%), mp 181—183 °C, $[\alpha]_D^{24}$ −13.3° (*c* 1.0, DMF), R_f^2 0.10, R_f^9 0.69. Amino acid ratios in acid hydrolyzate: Thr 0.95 (1), Ser 0.89 (1), Glu 1.07 (1), Ala 1.01 (1), His 1.04 (1), Lys 1.03 (1), NH_3 1.28 (1). Found: C, 57.12; H, 6.53; N, 12.14%. Calcd for $C_{54}H_{72}O_{14}N_{10}\cdot3H_2O$: C, 56.93; H, 6.90; N, 12.29%.

Boc-Leu-Lys(Z)-Pro-Leu-Ala-OH (10). To an ice-chilled solution of **9** (13.587 g) in methanol (15 ml) was added 1.98 M NaOH (9.57 ml). The mixture was stirred for 170 min at room temperature; then, 1 M HCl (18.95 ml) was added to this solution in an ice-bath. After evaporation of the methanol, the residual solution was dissolved in 1-butanol (ca. 150 ml) saturated with water, and the organic layer was washed with water (10 ml×3). After removing the solvent, the product was recrystallized from ethyl acetate-diethyl ether; 12.300 g (92%), mp 105—114 °C, $[\alpha]_D^{24}$

−77.2° (*c* 1.0, MeOH), R_f^6 0.44, R_f^7 0.81. Amino acid ratios in acid hydrolyzate: Pro 0.97 (1), Ala 0.98 (1), Leu 2.03 (2), Lys 1.02 (1). Found: C, 56.54; H, 7.80; N, 10.07%. Calcd for $C_{39}H_{62}O_{10}N_6 \cdot 3H_2O$: C, 56.51; H, 8.27; N, 10.14%.

Boc-Glu(OBzl)-Leu-Lys(Z)-Pro-Leu-Ala-OH (11). Compound **10** (3.100 g) was treated with TFA (20 ml)-anisole (0.87 ml) to give a solid. The solution of Boc-Glu(OBzl)-ONp (2.847 g) in DMF (10 ml) was mixed with the solution of the above-mentioned solid and Et_3N (1.11 ml) in DMF (20 ml). The mixture was stirred for 69 h at room temperature and then condensed. The residue was dissolved in 1-butanol saturated with water and the 1-butanol layer was washed with 5% citric acid (30 ml×1) and water (10 ml×1). After removing the solvent, the product was dissolved in DMF (ca. 20 ml) and the solution was applied to a Sephadex LH-20 column (2.7×140.5 cm) employing the same solvent as the eluate. Fractions of 10 g each were collected. Fractions 30—38, including the desired peptide, were combined and the solvent was removed. After the residue was extracted with ethyl acetate (200 ml), the organic layer was further washed in the same manner mentioned above and concentrated. Fractions 39—46, containing impurity, were combined and concentrated. After the addition of ethanol to the residue, the resulting precipitate was removed by filtration. An additional crop was obtained from the filtrate by rechromatography on the same column. The combined product was crystallized from diethyl ether; 3.433 g (86%), mp 92—96 °C, $[\alpha]_D^{24}$ −74.6° (*c* 1.0, MeOH), R_f^6 0.45, R_f^7 0.84, R_f^9 0.87. Amino acid ratios in acid hydrolyzate: Glu 1.05 (1), Pro 1.02 (1), Ala 0.93 (1), Leu 2.04 (2), Lys 0.97 (1). Found: C, 60.94; H, 7.72; N, 9.69%. Calcd for $C_{51}H_{75}O_{13}N_7 \cdot 1/2H_2O$: C, 61.06; H, 7.64; N, 9.77%.

Boc-His(Boc)-His-Glu(OBzl)-Ala-Opac (14a) in Fig. 3. Compound **13a** (3.914 g) was treated with TFA (22 ml) in the presence of anisole (2.23 ml). The solid obtained by the usual method showed two spots (R_f^{10} 0.73, 0.79) on TLC in an approximately equal proportions. The crude product was employed for the following coupling without purification. To an ice-cooled solution of Boc-His(Boc)-OH (2.185 g) in DMF (9.0 ml) were added HOBt (1.246 g), EDC·HCl (1.297 g), and the ice-chilled solution of the above solid and Et_3N (1.42 ml) in DMF (23 ml). The mixture was stirred for 44.5 h in an ice-bath and then at room temperature. After evaporation of the solvent the residue was extracted with ethyl acetate (200 ml), and the organic layer was washed using the usual procedure. The residue obtained after removing the solvent showed two spots on TLC (components A and B). This crude residue was dissolved in chloroform (30 ml) and the solution was subjected to a silica-gel column (2.2×35.0 cm) which was eluted with chloroform-methanol solvent systems. The fractions containing component A, Boc-His(Boc)-His-(Boc)-Glu(OBzl)-Ala-Opac (**14b**), were combined and the solvent was evaporated. The residue was triturated with diethyl ether; 1.305 g, mp 90—91 °C, $[\alpha]_D^{24}$ −22.0° (*c* 1.0, MeOH), R_f^1 0.47 (trace 0.26, 0.36), R_f^2 0.79 (trace 0.36, 0.66). Amino acid ratios in acid hydrolyzate: Glu 1.06 (1), Ala 1.01 (1), His 1.94 (2). Found: C, 59.80; H, 6.47; N, 11.15%. Calcd for $C_{50}H_{64}O_{14}N_8$: C, 59.99; H, 6.44; N, 11.19%. FAB mass: *m/z* 1001 (MH^+, $C_{50}H_{65}O_{14}N_8$). Component B, corresponding to the desired peptide, **14a**, was isolated from other fractions in the same chromatography described above; 0.965 g (21%), mp 86—87.5 °C, $[\alpha]_D^{24}$ −30.0° (*c* 1.0, MeOH), R_f^1 0.11 (trace 0.36), R_f^2 0.40 (trace 0.79). Amino acid ratios in acid hydrolyzate: Glu 1.04 (1), Ala 1.00 (1), His 1.96 (2). Found: C, 58.80; H, 6.40; N, 11.97%. Calcd for $C_{45}H_{56}O_{12}N_8 \cdot H_2O$: C, 58.81; H, 6.36; N, 12.19%. FAB mass: *m/z* 901 (MH^+, $C_{45}H_{57}O_{12}N_8$).

Boc-Lys(Z)-Lys(Z)-Lys(Z)-Gly-OEt (17). Compound **16** (24.047 g) was treated with TFA (30 ml)-anisole (3.59 ml), in the usual method, to give a solid. Boc-Lys(Z)-OH prepared from its dicyclohexylammonium salt (22.297 g) was dissolved in DMF (50 ml) and the solution was cooled below −20 °C. NMM (4.37 ml) and ECC (3.81 ml) were added to this solution with stirring. The mixture was stirred for 5 min at −14—−16 °C, and then cooled below −20 °C. To the mixture was added the ice-cooled solution of the above solid and Et_3N (4.60 ml) in DMF (50 ml). The mixture was stirred for 20.5 h in an ice-bath and then at room temperature. After the solvent was evaporated, the residue was extracted with ethyl acetate (300 ml). The solution was washed with water (300 ml×1). The precipitation brought about by the washing was dissolved by warming. The solution was dried and concentrated. The residue was recrystallized from methanol-chloroform-diethyl ether and chloroform-diethyl ether; 25.670 g (78%). For the purpose of analysis, a part of the product (0.320 g) was further purified by a silica-gel column (1.7×35.2 cm) with chloroform-methanol solvent systems as eluates. The obtained product was recrystallized from chloroform-methanol; 0.297 g (93%), mp 148.5—151.5 °C, $[\alpha]_D^{24}$ −14.3° (*c* 1.0, DMF), R_f^1 0.51, R_f^2 0.78. Amino acid ratios in acid hydrolyzate: Gly 1.00 (1), Lys 3.01 (3). Found: C, 61.26; H, 7.29; N, 9.80%. Calcd for $C_{51}H_{71}O_{13}N_7 \cdot 1/2H_2O$: C, 61.31; H, 7.26; N, 9.81%.

Boc-Lys(Z)-Lys(Z)-Lys(Z)-Gly-His-His-Glu(OBzl)-Ala-Opac (20). Compound **14a** (1.367 g) was treated with TFA (15 ml) and anisole (0.659 ml), in the usual way, to yield a solid (**19**), 3TFA·H-His-His-Glu(OBzl)-Ala-OPac; R_f^9 0.13, R_f^{10} 0.55, R_f^{11} 0.57. Found: C, 46.40; H, 4.01; N, 10.71%. Calcd for $C_{41}H_{43}O_{14}N_8F_9 \cdot H_2O$: C, 46.42; H, 4.28; N, 10.56%. The ice-chilled solution of **18** (1.824 g) in DMF (4.5 ml) was mixed with HOBt (0.384 g), EDC·HCl (0.436 g), and an ice-cooled solution of **19** and Et_3N (0.633 ml) in DMF (7.0 ml). The mixture was stirred for 18.5 h in an ice-bath and then at room temperature. The residue yielded, after removing the solvent, was dissolved in 1-butanol saturated with water. The organic layer was washed in the routine manner. After the solution was concentrated, the residue was recrystallized from methanol; 2.195 g (88%), mp 170 °C (decomp), $[\alpha]_D^{24}$ −18.8° (*c* 1.0, DMF), R_f^4 0.90, R_f^9 0.60. Amino acid ratios in acid hydrolyzate: Glu 1.08 (1), Gly 0.98 (1), Ala 0.97 (1), His 1.96 (2), Lys 3.00 (3). Found: C, 60.61; H, 6.57; N, 12.54%. Calcd for $C_{84}H_{105}O_{20}N_{15} \cdot H_2O$: C, 60.67; H, 6.49; N, 12.63%.

Boc-Lys(Z)-Lys(Z)-Lys(Z)-Gly-His-His-Glu(OBzl)-Ala-OH (21). Compound **20** (6.239 g) was dissolved in acetic acid (27 ml). Zinc powder (8.00 g) was added to this solution. The mixture was stirred for 4.5 h at 40 °C. After removing the catalyst, the filtrate was evaporated and the residue was triturated with water. The precipitates were collected and recrystallized from methanol-ethyl acetate; 4.861 g (84%), mp 176 °C (decomp), $[\alpha]_D^{24}$ −15.3° (*c* 1.0, DMF), R_f^9 0.53 (trace 0.44). Amino acid ratios in acid hydrolyzate: Glu 1.03 (1), Gly 1.01 (1), Ala 0.98 (1), His 1.98 (2), Lys 3.00 (3). Found: C, 59.04; H, 6.69; N, 13.52%. Calcd for $C_{76}H_{99}O_{19}N_{15} \cdot H_2O$: C, 59.09; H, 6.59; N, 13.60%.

Boc-Glu(OBzl)-Leu-Lys(Z)-Pro-Leu-Ala-Gln-Ser(Bzl)-His-Ala-Thr-Lys(Z)-OBzl (22). Compound **5** (4.070 g) was treated with TFA (40 ml)–anisole (0.815 ml) in the usual manner to produce a solid. To an ice-cooled solution of **11** (4.847 g) in DMF (12.5 ml) were added HOBt (0.988 g), EDC·HCl (1.028 g) and the ice-chilled solution of the above solid and Et_3N (1.04 ml) in DMF (17.5 ml). The mixture was stirred for 72 h in an ice-bath and then at room temperature. The solvent was evaporated, the residue was dissolved in 1-butanol (ca. 150 ml) saturated with water and the organic layer was washed in the usual method. After concentration in vacuo, the crude product was dissolved in DMF (10 ml) and the solution was subjected to a Sephadex LH-60 column (2.2×143.8 cm) using the same solvent as the eluate. Fractions of 10 g each were collected. Fractions 28—53 were combined and the solvent was evaporated. The residue was recrystallized from methanol; 6.300 g (86%), mp 192 °C (decomp), $[\alpha]_D^{24}$ −31.8° (*c* 1.0, DMF), R_f^4 0.99 (HPTLC), R_f^9 0.75 (HPTLC). Amino acid ratios in acid hydrolyzate: Thr 0.84 (1), Ser 0.79 (1), Glu 2.11 (2), Pro 1.16 (1), Ala 1.91 (2), Leu 2.31 (2), His 0.91 (1), Lys 1.98 (2), NH_3 1.07 (1). Found: C, 60.19; H, 7.31; N, 11.71%. Calcd for $C_{100}H_{137}O_{24}N_{17}\cdot 2H_2O$: C, 60.14; H, 7.12; N, 11.92%.

Boc-Lys(Z)-Lys(Z)-Lys(Z)-Gly-His-His-Glu(OBzl)-Ala-Glu(OBzl)-Leu-Lys(Z)-Pro-Leu-Ala-Gln-Ser(Bzl)-His-Ala-Thr-Lys(Z)-OBzl (23). Compound **22** (3.000 g) was treated with TFA-CH_2Cl_2 (1:1) (40 ml) containing anisole (0.498 ml) in the usual procedure to afford a solid. Compound **21** (2.803 g) was dissolved in DMF (12 ml) and the solution was cooled in an ice-bath. The solution was mixed with HOBt (0.372 g), EDC·HCl (0.387 g), and the ice-cooled solution of the above solid and Et_3N (0.475 ml) in DMF (19 ml). The mixture was stirred for 65 h in an ice-bath and then at room temperature. The solvent was evaporated and the residue was dissolved in 1-butanol saturated with water. The 1-butanol layer was washed using the routine method. After removing the solvent, the product was dissolved in DMF (20 ml), and the solution was applied to a column (2.2×140 cm) of Sephadex LH-60 using the same solvent as the eluate. Fractions of 10 g each were collected. Fractions 26—42 were combined and the solvent was evaporated. The residue was repeatedly chromatographed on the same column, and recrystallized from methanol. However, the product still contained minor impurities; 2.856 g (55%). For the sake of further purification for analysis, the product (0.500 g) was dissolved in 1-butanol-acetic acid-water (4:1:1) (2.0 ml) and applied to a column (1.5×38.5 cm) of silica gel (Silica gel 100; Merck and Co.), which had been equilibrated with the same solvent system. It was eluted with the same solvent. Fractions of 5.0 g each were collected. Fractions 17—29 were combined and the solvent was removed. The recrystallization of the residue from methanol afforded a pure product; 0.122 g (24%), mp 198 °C (decomp), $[\alpha]_D^{24}$ −15.9° (*c* 1.0, DMF), R_f^3 0.83, R_f^8 0.67, R_f^9 0.60. Amino acid ratios in acid hydrolyzate: Thr 0.91 (1), Ser 0.87 (1), Glu 3.04 (3), Pro 1.19 (1), Gly 1.00 (1), Ala 2.95 (3), Leu 2.03 (2), His 2.99 (3), Lys 5.01 (5), NH_3 1.19 (1). Found: C, 59.55; H, 6.81; N, 12.93%. Calcd for $C_{171}H_{226}O_{40}N_{32}\cdot 4H_2O$: C, 59.67; H, 6.85; N, 13.02%.

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