Synthesis and Redox Property of Polypeptides Containing L-Ferrocenylalanine

Michie Kira,† Teruhiko Matsubara,† Hiroaki Shinohara,†,†† and Masahiko Sisido*†

†*Department of Bioscience and Biotechnology, Faculty of Engineering, Okayama University, 3-1-1 Tsushimanaka, Okayama 700*

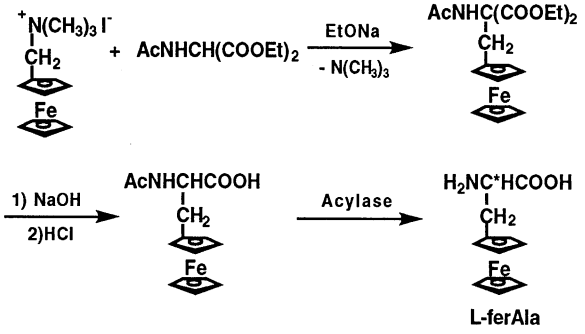
††*PRESTO, Japan Science and Technology Corporation, 3-1-1 Tsushimanaka, Okayama 700*

(Received September 17, 1996)

An optically-active redox amino acid, L-ferrocenylalanine was synthesized. The amino acid was incorporated into polypeptides in the form of a single L-ferrocenylalanine unit or a L-ferrocenylalanyl-L-ferrocenylalanine unit. Those polypeptides showed reversible redox property on the cyclic voltammetry.

Ferrocenyl group has been known to mediate electron transfers between electrodes and redox sites of proteins. By attaching ferrocenyl groups onto proteins, redox activity of proteins has been driven or regulated by external potentials. Several workers reported attempts to modify redox proteins with ferrocene derivatives under relatively drastic conditions.[1] In the chemical modifications however, neither the number of ferrocenyl groups nor their positions is specified in the protein. To obtain full redox activity, one must incorporate ferrocenyl groups into specific sites of the proteins.

Site-specific incorporation of redox-active nonnatural amino acids into proteins has become possible through chemical synthesis of proteins[2] or through extension of genetic engineering technique.[3] In both approaches, the first key step is the synthesis of nonnatural amino acids carrying a ferrocenyl side groups. In this report, synthesis and optical resolution of L-ferrocenylalanine (ferAla) and preparation of polypeptides that contain ferAla are described. Synthesis of racemic ferAla through an N-formyl derivative has been reported by Osgerby and Pauson.[4] We have improved their route to synthesize acetyl DL-ferAla for the ease of subsequent optical resolution with acylase. The synthetic route is shown in the Scheme below.



Sodium (0.6 g, 0.026 mol) was dissolved in dry ethanol (100 mL), then diethyl acetamidomalonate (5.64 g, 0.026 mol) and subsequently ferrocenylmethylammonium iodide (5.0 g, 0.013 mol) were added to the solution. The mixture was refluxed at 90 °C for 20-80 h until the evolution of trimethylamine was ceased. Ethanol was removed under reduced pressure and water was added. The organic component was extracted with ether and dried over Na_2SO_4. Evaporation of ether gave an oily product (diethylferrocenylmethylacetamidomalonate).

The latter (3.0 g, 7.2 mmol) was dissolved in ethanol (30 mL) and a 4-fold excess of 1N NaOH was added. The mixture was refluxed for 3 h and cooled to room temperature. The mixture was acidified to pH=2 with HCl and refluxed again for 2 h. The ethanol was evaporated and the product, (N-acetyl-DL-ferrocenylalanine) was precipitated with water. Yield 1.85g (82% from the diethyl ferrocenylmethylacetamidomalonate), mp 198 °C. ^{1}H NMR (DMSO-d_6+1%TFA) d=1.80 (3H, s, Ac), 2.6-2.9 (2H, br. C$^\beta$H2), 4.06 (9H, s, ferrocenyl rings), 4.20 (1H, C$^\alpha$H), 8.1 (1H, d, NH).

The racemic acetylamino acid was dissolved in 1N NaOH and the pH was adjusted to 7 with HCl and acylase solution (acylase from Aspergillus genus, 260 mg in water 10 mL with 4 mg of $CoCl_2 \cdot 6H_2O$) (4 mL) was added and gently stirred at 37 °C for 2 days. L-FerAla was precipitated as yellow powder and recrystallized from methanol. Yield 0.55 g (71% from the acetyl derivative).

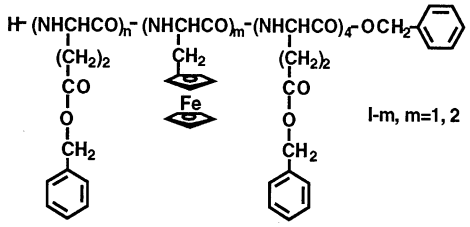
The optical purity of ferAla after the selective deacetylation with acylase was checked by the diastereomer formation with (S)-(-)-1-(2,3-naphthalenedicarboxyimidyl)propionyl fluoride (NIPF, Dojin). The amino acid (1 mg) was suspended in DMF and 100 μL of the supernatant was mixed with the NIPF solution in acetonitrile (3.4 mM, 100 μL). After standing the solution for 4h at room temperature, the diastereomer mixture was analyzed by HPLC (ODS column). Under conditions where diastereomers from DL-phenylalanine showed a doublet, only single peak was detected, indicating that the ferAla obtained from the acylase reaction is optically pure.

L-Ferrocenylalanine (0.30 g, 1.1 mmol) was suspended in dioxane/water (2/1) mixture (3 mL) and $NaHCO_3$ (0.23 g) in water (1.5 mL) was added. Di-t-butyldicarbonate [$(Boc)_2O$, 0.29 g, 1.32 mmol] in dioxane (1 mL) was added dropwise under cooling with ice-water. The mixture was stirred for 12 h and evaporated to remove dioxane. The pH was adjusted to 2 with 5% $KHSO_4$ and the oil appeared was extracted with ethyl acetate. The extract was dried over Na_2SO_4 and evaporated to give Boc-L-ferAla. Yield 0.25 g (61%). ^{1}H NMR (CD_3OD) d=1.40 (9H, s, tBu), 2.6-2.9 (2H, br. C$^\beta$H2), 4.1 (1H, s, C$^\alpha$H), 4.15 (9H, s, ferrocenyl rings). Found: C, 57.80, H; 6.09; N, 3.68%. Calcd for $C_{18}H_{23}NO_4Fe$: C, 57.93; H, 6.21; N, 3.75%.

Absorption and CD spectra of Boc-L-ferAla were measured in trimethyl phosphate (TMP) at 4.2×10^{-3} M for longer wavelengths and at 8.4×10^{-6} M for shorter wavelengths. The spectra are shown in Figure 1. Concentrations of the peptides containing ferAla were determined from the absorption coefficient at 435.5 nm (ε=103) and at 203.5 nm (5.07×10^4) in the following experiments.

L-Ferrocenylalanine was incorporated into helical poly(γ-benzyl L-glutamate), poly[Glu(OBzl)], in the form of I-m. A pentapeptide, Boc-ferAla-Glu(OBzl)$_4$-OBzl, was synthesized by a conventional liquid-phase method from HCl·Glu(OBzl)$_4$-OBzl and Boc-ferAla, using EDC·HCl/HOBt /triethylamine in DMF.[2]

The pentapeptide was purified by a preparative HPLC with an ODS column in acetonitrile until a single peak was attained.



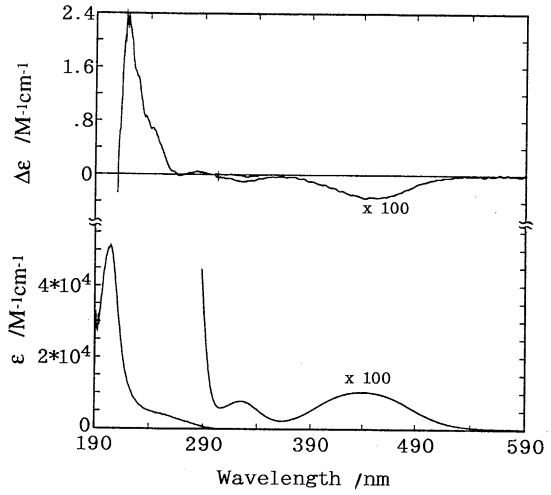


Figure 1. Absorption (bottom) and CD (top) spectra of N-t-butyloxycarbonyl-L-ferrocenylalanine in trimethyl phosphate

The Boc group was removed in 4N HCl/dioxane and the HCl·peptide salt was neutralized with 4% $NaHCO_3$ solution. The pentapeptide with free amino group was used as an initiator for the polymerization of N-carboxy-anhydride of Glu(OBzl) at the [NCA]/[amino group] ratio of 40. After 4 days, the polypeptide was precipitated into ether and collected.

Absorption spectrum of the polypeptide was measured in TMP. The spectrum consisted of the contributions from phenyl group, from amide group, and from ferrocenyl group at longer wavelengths than 280 nm. From the analysis of the spectrum, the degree of polymerization of the Glu(OBzl) units, n, was calculated to be 59.

CD spectrum of the polypeptide was also measured in TMP. The spectrum showed typical pattern of right-handed α-helix conformation at the amide absorption region. The Δε value at 222 nm was -12.5, indicating fully helical conformation. CD peaks that can be assigned to ferrocenyl group could not be detected, probably because of the small Δε value.

A hexapeptide containing ferAla-ferAla sequence, Boc-ferAla$_2$-Glu(OBzl)$_4$ was also prepared and used as an initiator for the polymerization of Glu(OBzl) NCA. In this case, the hexapeptide was synthesized through solid-phase method using oxime-resins.[5] Boc-ferAla$_2$-Glu(OBzl)$_3$ was prepared on the oxim resin and cleaved off by adding 3-fold excess of Glu(OBzl)·TosOH. The resulting Boc-hexapeptide was purified by preparative HPLC with ODS column, and it was deprotected with HCl/ dioxane and used as initiator of Glu(OBzl) NCA as before. CD spectra of the latter polypeptide also showed typical pattern of right-handed α-helix. The CD data show that the polypeptides take α-helical conformation despite the incorporation of bulky ferAla units.

Electrochemical behavior of the polypeptides was studied with cyclic voltammetry in DMF solution under nitrogen atmosphere. A glassy carbon disk electrode (ϕ=3 mm), a platinum plate, and a Ag/Ag$^+$ (0.01M $AgNO_3$+0.1 M nBu_4NBF_4) electrode were used as a working electrode, a counter electrode, and a reference electrode, respectively. Cyclic voltammograms of the two polypeptides and Boc-ferAla are shown in Figure 2.

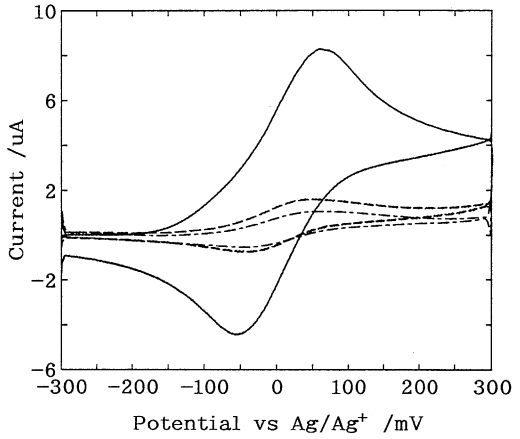


Figure 2. Cyclic voltammograms of Boc-ferAla(——), polypeptide I-1(-----) and polypeptide I-2 (—·—) in 0.1M nBu_4NBF_4/DMF. [ferAla]=0.5 mM, Scan rate=100 mVs^{-1}.

The two polypeptides show reversible redox peaks at about the same potentials as that of Boc-ferAla, although the peak intensities are reduced due to the slow diffusion rates and possibly due to the shielding by the benzyl side groups. . The similar cyclic voltammograms for the polypeptides I-1 and I-2 indicate that the ferrocenyl groups in the I-2 polypeptide are electronically independent to each other as the isolated ferrocenyl group in the polypeptide I-1.

To conclude, L-ferAla is a novel amino acid that can add artificial redox functions to synthetic polypeptides and, possibly, to genetically engineered proteins.

This work was supported by a Grant-in-Aid for Scientific Research on Priority Area (No.236) "New Development of Organic Electrochemistry" from the Ministry of Education, Science, Sports, and Culture.

References and Notes

1 a) Y. Degani and A. Heller, *J. Phys. Chem.*, **91**, 1285 (1987), b) Y. Degani and A. Heller, *J. Am. Chem. Soc.*, **110**, 2615 (1988).
2 N. Izumiya, T. Kato, H. Aoyagi, and M. Waki, "Pepuchido Gousei no Kiso to Jikken (in Japanese)", Maruzen, Tokyo, (1985).
3 a) C.J. Noren, S.J. Anthony-Cahill, M.C. Griffith, and P.G. Schultz, *Science* , **244**, 182 (1989), b) J.D. Bain, C.G. Glabe, T.A. Dix, A.R. Chamberlin, and E.S. Diala, *J. Am. Chem. Soc.*, **111**, 8013 (1989), c) T. Hohsaka, K. Sato, M. Sisido, K. Takai, and S. Yokoyama, *FEBS Lett.*, **344**, 171 (1994).
4 J.M. Osgerby and P.L. Pauson, *J. Chem. Soc.*, **1958**, 656.
5 H. Mihara and N. Nishino, *J. Synth. Org. Chem. Jpn.*, **52**, 50 (1994).