Influence of Anhydrous Hydrogen Fluoride on Hen Egg-White Lysozyme. II. Effects on Reduced Hen Egg-White Lysozyme

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The influences of anhydrous hydrogen fluoride on reduced hen egg-white lysozyme were investigated under standard conditions for peptide synthesis. 1) Enzymatically fully active protein was recovered in poor yield (5%) from reduced hen egg-white lysozyme after treatment with hydrogen fluoride. 2) The yield (29—33%) was improved by addition of 1,4-butanedithiol or L-cysteine during treatment of reduced hen egg-white lysozyme with hydrogen fluoride. 3) Tetragonal crystals which were indistinguishable from those of native hen egg-white lysozyme were recovered after treatment of reduced hen egg-white lysozyme with hydrogen fluoride in the presence of 1,4-butanedithiol, anisole and amino acid derivatives. Thus, the original intact lysozyme could be recovered from an HF-solution of the reduced form.

In studies on the chemical synthesis of the well-characterized protein molecule, native hen egg-white lysozyme (HEL), we examined its recovery after exposure of this enzyme and its derivatives to anhydrous liquid hydrogen fluoride (HF) under usual conditions for peptide synthesis. In the previous paper[1] we reported the effect of HF on the native form of HEL under usual conditions of peptide synthesis and a method for recovering fully active HEL from an HF-solution of native HEL.

This paper reports studies on the influence of HF on the reduced form of HEL. It was found that when reduced HEL was treated with HF under usual conditions for peptide synthesis, its enzymatic activity decreased. In contrast when native HEL was treated with HF, fully active HEL was recovered in good yield (about 50%). Thus there was a difference in the effects of HF on the native and reduced forms of HEL. Next, the effect of thiol compounds during treatment of reduced HEL with HF was examined, because thiol compounds prevent modifications, such as oxidation of proteins. Studies were also made on the effect of treating reduced HEL with HF in the presence of amino acid derivatives, which liberate cations on treatment with HF. Anisole has generally been used as a scavenger of cations liberated from protected amino acids and peptides in acidic medium[2] or on treatment with HF[3]. Therefore, we also examined the effect of the presence of anisole in preliminary experiments on deblocking reactions of protected derivatives[4] of HEL. The results showed that intact, regenerated HEL could be recovered after treating reduced HEL with HF under conditions for peptide synthesis.

Experimental

Materials. Hen egg-white lysozyme (6×crystallized Lot. No. 7103) and spray-dried cells of *Micrococcus lysodeikticus* (Lot. No. 21—88—778) were purchased from Seikagaku Biochemicals (Tokyo). Sephadex was purchased from Pharmacia Co. (Uppsala). Hydrogen fluoride was a product of Daikin Ind. Co. (Osaka). Urea of reagent grade was recrystallized from ethanol before use. All other chemicals used were of analytical grade.

Treatment of Reduced HEL with HF. Reduced HEL was prepared from native HEL by the method of Saxena and Wetlaufer,[5] and after desalting, it was frozen and lyophilized. The freeze-dried powder of reduced HEL (*ca.* 15 mg) was treated with HF (9 ml) under similar conditions to those used with native HEL.[1] The residue of the HF-treated reduced HEL was dissolved in 0.2 M sodium phosphate buffer solution (15 ml) at pH 8.5 containing 1 M mercaptoethanol, 8 M urea and 0.2 mM EDTA·2Na and the solution was stirred overnight at room temperature. Then it was adjusted to below pH 4 by addition of glacial acetic acid and passed through a column of Sephadex G-10 (3.2×60 cm) in 1 M acetic acid. The fractions containing protein were collected and treated under the conditions described below for refolding reduced HEL. Then, the solution containing regenerated HEL was adjusted to pH 4.5—5 with glacial acetic acid. The regenerated HEL was adsorpted on a CM-cellulose column (carboxylic acid cycle), and eluted with 0.3 M sodium phosphate buffer solution at pH 8.0.

Refolding of Reduced HEL to Native HEL. Refolding of reduced HEL (*ca.* 10^{-6} M) to form native HEL was achieved in 0.08 M tris-buffer solution at pH 8.0 containing 5.4×10^{-3}M L-cysteine and 4.8×10^{-4} M L-cystine at 37 °C following the procedure of Saxena and Wetlaufer.[5]

Gel-filtration and Ion-exchange Chromatography of HF-treated Reduced HEL. HF-treated HEL refolded as described above was chromatographed on Sephadex G-50 (2×95 cm) using buffer containing 1M acetic acid and 5 M urea, using the method described in the previous paper.[1] The fractions separated by gel-filtration on Sephadex G-50 were chromatographed on Bio-rex 70 (1×78 cm) using 0.2 M sodium phosphate buffer solution at pH 7.18.

Enzyme Assays. The specific activity of HF-treated HEL was determined by measuring the initial rate of lysis of *Micrococcus lysodeikticus* cells spectrophotometrically at 540 nm following the method described previously,[1] with native HEL, purified on Sephadex G-50, as a standard.

Ultraviolet Spectra. UV-absorption spectra were measured using a Hitachi-spectrophotometer, type-124, equipped with a recording attachment. Spectra were measured at 25 °C.

Crystallization of HF-treated HEL. Crystallization was carried out by the method of Berthou and Jolles[6] with the modifications described in the previous paper.[1]

Results and Discussion

First reduced HEL was treated with HF at 0 °C for 60 min, since it has been found that intact HEL is recovered in good yield (about 50%), when native HEL is treated with HF under these conditions. The HF was completely evaporated off from the HF-solution

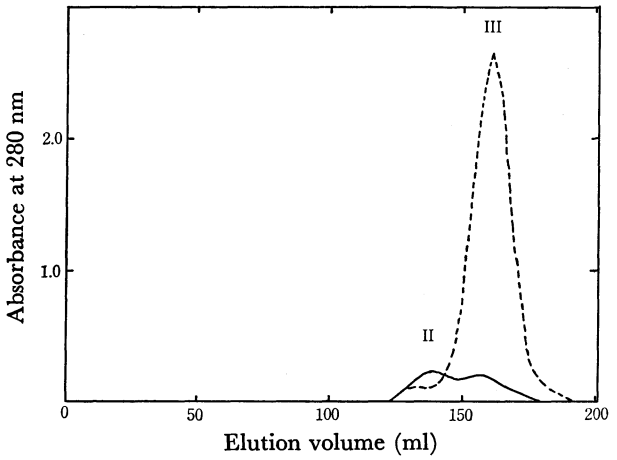

Fig. 1. Sephadex G-50 gel-filtration of reduced HEL after treatment with HF at 0 °C for 60 min and refolding at pH 8.0. The column (2.2×95 cm) was eluted with buffer containing 1M acetic acid and 5M urea. The flow rate was 10 ml per hr.
—: reduced HEL refolded after HF-treatment, ---: reduced HEL refolded without HF-treatment.

of reduced HEL *in vacuo*, and the residue was mixed with 0.2 M sodium phosphate buffer solution at pH 8.5 containing 1 M mercaptoethanol, 8 M urea and 0.2 mM EDTA·2Na. This buffer was used because it was thought that reduced HEL might aggregate by forming random disulfide linkages in HF and that this buffer solution might dissociate the aggregates, because it has been used for reducing native enzyme.[5] In fact the residue did aggregate. However, the aggregates did not easily dissolve in this buffer solution. The supernatant was separated from aggregated material and desalted and then treated under the conditions used for refolding reduced HEL. The refolded protein was desalted, lyophilized and chromatographed on Sephadex G–50 in 1 M acetic acid and 5 M urea buffer solution, as shown in Fig. 1. The recovery of a fraction (named fraction III), eluted in the same position as native HEL, corresponded to only 5% of the original native HEL. The recovery of this fraction was low probably because reduced HEL aggregated to sparingly soluble material on treatment with HF.

Next, reduced HEL was treated with HF under the same conditions but in the presence of the thiol compounds listed in Table 1. The residue of HF-treated reduced HEL dissolved in 0.2 M sodium phosphate buffer solution containing 1 M mercaptoethanol, 8 M urea and 0.2 mM EDTA·2Na. This protein fraction was desalted by gel-filtration on Sephadex G-10, treated under the conditions leading to refolding of reduced HEL and then chromatographed on Sephadex G-50. It was found that the recovery of original HEL, eluted in the same position as native HEL, was improved considerably using L-cysteine or 1,4-butanedithiol as the thiol compound and was improved slightly using dithiothreitol. However, scarcely any fraction III was obtained using 2-mercaptoethanol. The chromatogram of regenerated protein on Sephadex G–50 obtained using 1,4-butanedithiol as a thiol compound is shown in Fig. 2. The amount of fraction III in Fig. 2 corresponds to the recovery shown in Table 1.

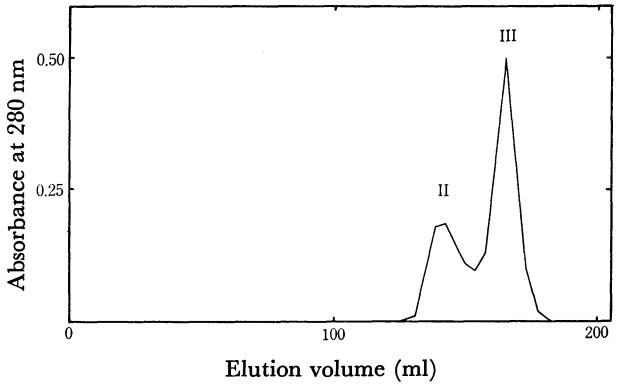

Fig. 2. Sephadex G-50 gel-filtration of reduced HEL after treatment with HF at 0 °C for 60 min in the presence of 1,4-butanedithiol and refolding at pH 8.0. The column (2.0×95 cm) was eluted with buffer containing 1M acetic acid and 5M urea. The flow rate was 10 ml per hr.

TABLE 1. EFFECTS OF THIOL COMPOUNDS ON THE RECOVERY OF FRACTION III FROM HF-TREATED REDUCED HEL

Exp. No.	Thiol compound	HF-treatment	Recovery of fraction III (%)	Specific activity[a] of fraction III (%)
1	—	—[b]	65	98
2	—	0 °C, 60 min	5	95
3	$HSCH_2CH_2OH$[c]	0 °C, 60 min	0—1	43
4	L-Cysteine·HCl ·H_2O[c]	0 °C, 60 min	24	97
5	L-Cysteine[c]	0 °C, 60 min	29	98
6	1,4-Butane-dithiol	0 °C, 60 min	33	99
7	Dithiothreitol[d]	0 °C, 60 min	19	100

a) 6x recrystallized HEL purified on Sephadex G-50 was used as a standard, b) reduced HEL was refolded without HF-treatment, c) 0.8 mmole of thiol compound was added per μmol of protein, d) 0.4 mmole of thiol compound was added per μmol of protein.

Next, we made preliminary experiments on the effect of removal of protecting groups from protected derivatives[4] of HEL. Reduced HEL was first treated with HF in the presence of anisole. 1,4-Butanedithiol was also added to the reaction mixture, because among the thiol compounds tested it caused the greatest improvement in recovery of the original enzyme, as shown in Table 1. When reduced HEL was treated with HF at 0 °C for 60 min with 1,4-butanedithiol, the recovery of the original enzyme was 14% in the presence of anisole and 33% in its absence. The reason for this lower recovery in the presence of anisole seemed to be that the excess anisole could not easily be removed from the HF-reaction mixture, so that the HF could not be removed rapidly. On increase in the time of reaction (120—240 min) of reduced HEL with HF at 0 °C, the amount of fraction III decreased. Furthermore, the yield of fraction III was very low when reduced HEL was treated with HF at a higher temperature (25 °C), even after reaction for only 30 min. These results are summarized in Table 2.

Table 2. Recoveries (%) of fraction III, from reduced HEL (15 mg) treated with HF (9 ml) under various conditions in the presence of 1,4-butanedithiol (0.3 ml) and anisole (1 ml), refolded at pH 8.0 and chromatographed on Sephadex G-50

	30 min	60 min	120 min	240 min
0 °C	17	14	4.1	2.4
25 °C	1.1	0.5	0.6	0.3

Reduced HEL was treated with HF in the presence of anisole, 1,4-butanedithiol and amino acid derivatives, which bind protecting groups and liberate them as cations on treatment with HF. The amino acid derivatives used were N^{ε}-benzyloxycarbonyl-L-lysine, N^{α}-benzyloxycarbonyl-*S*-benzyl-L-cysteine and *S*-*p*-methoxybenzyl-L-cysteine which liberate the benzyl group on treatment with HF, N^{α}-benzyloxycarbonyl-N^{ω}-tosyl-L-arginine which liberates a tosyl group, *t*-butyloxycarbonyl-glycine which liberate a *t*-butyl group and N^{ω}-nitro-L-arginine which liberates a nitro group. The results in Table 3 show that most of these amino acid derivatives did not affect the yield of fraction III but N^{ω}-nitro-L-arginine decreased the yield greatly. The reason for this is unknown.

Table 3. Effects of various amino acid derivatives on the recovery of fraction III from HF-treated reduced HEL

Exp. No.	Amino acid derivative	(mmole)[a]	Recovery of fraction III (%)
1	H-Lys(Z)-OH	1.0	13
2	Z-Cys(Bzl)-OH	0.5	15
3	H-Cys(Bzl(OMe))-OH	1.0	13
4	Z-Arg(Tos)-OH	1.0	16
5	Boc-Gly-OH	1.0	14
6	H-Arg(NO_2)-OH	1.0	3

a) Reduced HEL (15 mg), 1,4-butanedithiol (0.3 ml), anisole (1 ml) and the indicated amino acid derivatives (0.5 mmol or 1.0 mmol) were mixed and treated with HF (9 ml) at 0 °C for 60 min.

Reduced HEL was treated with HF 0 °C for 60 min in the presence of the mixture of amino acid derivatives given in Fig. 3. The regenerated protein fraction was chromatographed on Sephadex G-50 using buffer solution containing 1 M acetic acid and 5 M urea, as shown in Fig. 3. The amount of fraction III obtained was 33% of the initial amount of enzyme. This yield was better than those obtained on treatment of reduced HEL with HF in the presence of any of the amino acid derivatives alone. When fraction III of Fig. 3 was subjected to chromatography on Bio-rex 70 at pH 7.18, the main fraction eluted in the position of native HEL (Fig. 4) was fully active on *Micrococcus lysodeikticus* cells, and it had the same UV-spectrum as native HEL, as shown in Fig. 5. During this chromatography, a fairly large fraction with biological activity was eluted in front of the main fraction, but its properties were not examined. The main fraction in Fig. 4 was desalted on Sephadex G-10 and lyophilized. The lyophilized

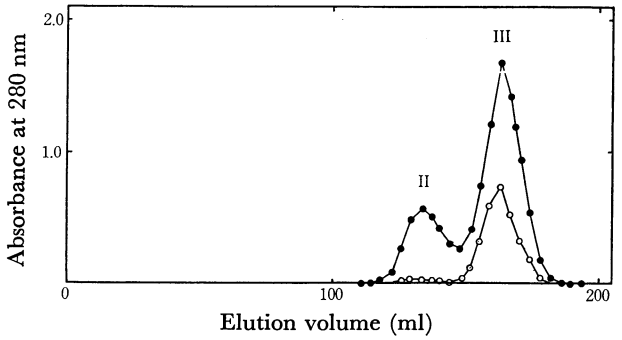

Fig. 3. Sephadex G-50 gel-filtration of reduced HEL treated with HF. The column (2.0×95 cm) was eluted with buffer containing 1M acid acetic and 5M urea. Reduced HEL (38 mg) was mixed with anisole (1.5 ml, 14 mmol), 1,4-butanedithiol (1.0 ml, 8.8 mmol), Z-Arg(Tos)-OH (231 mg, 0.5 mmol), H-Cys (Bzl)-OH (106 mg, 0.5 mmol), H-Cys(Bzl(OMe))-OH·HCl (130 mg, 0.5 mmol) and Boc-Gly-OH (88 mg, 0.5 mmol), treated with HF (15 ml) and refolded at pH 8.0. -●-: HF-treated reduced HEL, -○-: Native HEL.

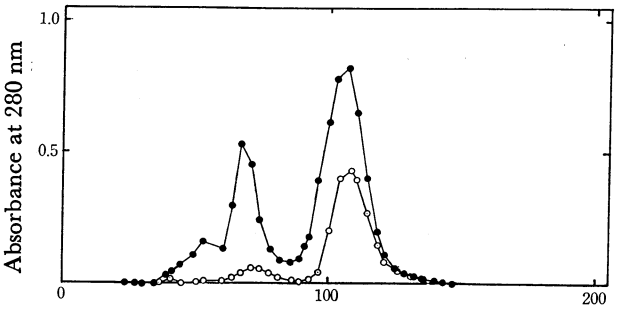

Fig. 4. Ion-exchange chromatogram of HEL on Bio-rex 70. The column (1×78 cm) was eluted with 0.2 M sodium phosphate buffer solution (pH 7.18). The flow rate was 16 ml per hr.
-●-: Fraction III of Fig. 3, -○-: Native HEL.

Table 4. Effects of various compounds on the recovery of fraction III from HF-treated native HEL

Compound	Average recovery of fraction III (%)	Ratio
1) —[a]	65	100
2) None	25	38
3) 1,4-Butanedithiol[b]	36	55
4) L-Cysteine[b]	32	49
5) L-Cysteine·HCl·H_2O[b]	32	49
6) $HSCH_2CH_2OH$[b]	0	0
7) L-Methionine[b]	34	52
8) L-Alanine[c]	26	40
9) L-Tryptophan[d]	18	28
10) L-Tryptophan[e]	3	5
11) L-Tryptophan[f]	0	0

a) Native HEL was reduced and refolded without HF-treatment. The amounts of compounds added per μmole of native HEL were: b) 2.5 mmol, c) 4.0 mmole, d) 0.5 mmol, e) 1.8 mmol and f) 2.4 mmol.

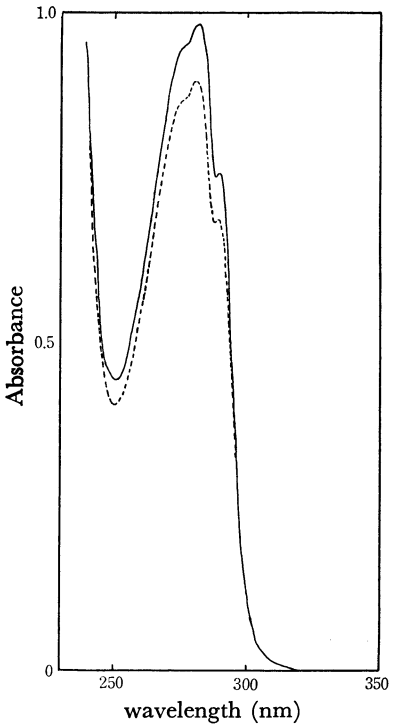

Fig. 5. Ultra-violet absorption spectra of the main fraction of Fig. 4 (—) and native HEL (---). Solvent: 0.2 M sodium phosphate buffer (pH 7.18).

powder was treated under conditions inducing crystallization. The crystals obtained were like those of native HEL, as shown in Fig. 6.

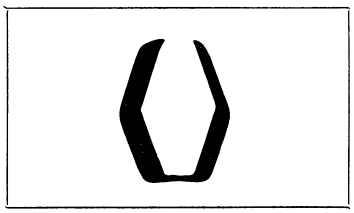

Fig. 6. Microphotograph of crystals of the material from the main fraction of Fig. 4. Crystals were obtained by the method of Berthou and Jolles.[6]

Native HEL was treated with HF under the same conditions as reduced HEL in the presence of various thiol compounds. After treatments similar to those used with reduced HEL the regenerated protein was subjected to chromatography on Sephadex G-50 using buffer solution containing 1 M acetic acid and 5 M urea. The yields of fraction III, eluted at the same position as native HEL, are summarized in Table 4. The recovery of the original enzyme from native HEL treated with in the presence of 1,4-butanedithiol and L-cysteine were 32 and 36%, respectively, while the recovery of original enzyme from native HEL treated with HF in the absence of thiol compounds was 25%. Thus thiol compounds also improved the yield on treatment of native HEL with HF. Since native HEL aggregates slightly in HF,[1] the thiol compounds seemed to prevent both this aggregation and also structural modification of HEL. It was also found that the presence of L-tryptophan during treatment of native HEL with HF greatly decreased the recovery of the original enzyme under the present conditions (Table 4).

The present results show that material with similar properties to original HEL could be recovered in fairly good yield and also in a crystalline state after treatment of reduced HEL with HF in the presence of a thiol compound such as 1,4-butanedithiol under usual conditions for peptide synthesis. In further studies on the chemical synthesis of HEL attempts are in progress to recover HEL with the same properties as native HEL from the *N*-benzyloxycarbonyl derivative of native HEL and the *S*-*p*-methoxybenzyl-derivative of reduced HEL.[4]

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