Deamidation at Asparagine-88 in Recombinant Human Interleukin 2

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Reversed-phase high-performance liquid chromatography (RP-HPLC) and protein-chemical analysis revealed that only Asn^{88} in recombinant human interleukin 2 (rIL-2) is liable to be deamidated during a long period of storage in aqueous solutions (pH 5.0) at 25 °C, even though there are eight asparagine and six glutamine residues. The deamidation occurred more easily at 40 °C than at 25 °C but did not occur at all at temperatures below 5 °C. The biological activity of Asn^{88}-deamidated rIL-2 was found to be almost the same as that of intact rIL-2, whereas its isoelectric point (pI 7.6) is different from that of intact rIL-2 (pI 7.9).

Keywords recombinant human interleukin 2; stability; deamidation; asparagine residue; RP-HPLC; peptide mapping

Human interleukin 2 (IL-2) is a lymphokine produced by T cells stimulated by antigens or mitogens and is well known as a proliferative and/or differentiative factor[1] of activated T cells, activated B cells and natural killer cells. The advent of deoxyribonucleic acid (DNA) cloning technology has allowed for the production of recombinant human interleukin 2 (rIL-2) having an alanine residue at the amino terminus, which is the same as natural IL-2, in *Escherichia coli*,[2] and this product has been highly purified. A number of pharmaceutical products such as interferon-αA,[3] granulocyte colony-stimulating factor,[4] and tissue-type plasminogen activator,[5] as well as rIL-2 which is currently being developed as an immunomodulator, have been produced by DNA cloning technology. Therefore, the elucidation of the long-term stability of recombinant proteins will play an important role in their successful application to pharmaceutical products. In the case of rIL-2, the chemical stability in aqueous solutions with the exception of disulfide scrambling[6] in alkaline solutions and aggregation[7] is not yet fully understood. We previously revealed[8] that Met^{104} in rIL-2 is easily oxidized to methionine sulfoxide in dilute solutions and clarified the susceptibility of oxidation among the four methionine residues in the molecule. We report here that Asn^{88} is slowly deamidated in aqueous solutions of rIL-2, unless the temperature is kept below 5 °C.

Materials and Methods

Materials rIL-2 was prepared and purified by the method previously described with some modification,[2,9] and the purified preparation was confirmed not to be deamidated. The final preparation of rIL-2 was a solution in 5 mM ammonium acetate (pH 5.0) containing 1 mg of protein per ml. Its biological activity was 1.27×10^7 JRU/mg protein (JRU, Japan reference unit established by the National Institute of Health (Japan)).

An unknown component (D-1) was purified by reversed-phase high-performance liquid chromatography (RP-HPLC) after the final preparation of rIL-2 was kept for 2 months at 40 °C. The purity was estimated to be 92% on the basis of the peak-area ratio on RP-HPLC.

A protein standard kit from Bio-Rad Laboratories was used to determine the molecular weight.

Achromobacter protease I,[10] tris-(hydroxymethyl)aminomethane (Tris) and urea were of biochemical grade, and the other reagents were of analytical grade from Wako Pure Chemical Industries, Ltd., unless stated otherwise.

RP-HPLC RP-HPLC was performed with an LC-4A chromatograph (Shimadzu Corp.). rIL-2 and D-1 were chromatographed using a Nucleosil $5C_{18}$ column (4 mm × 30 cm) (Macherey Nagel Co.). Elution was performed with a linear gradient from 47.5% (v/v) to 62.5% (v/v) acetonitrile (MeCN) in 0.1% (v/v) trifluoroacetic acid (TFA) over 60 min with a flow rate of 0.7 ml/min. The flow rate was changed to 1.0 ml/min using a Nucleosil $5C_{18}$ column (8 mm × 30 cm) for isolation.

The enzymatic digests of rIL-2 and D-1 were eluted at 0.7 ml/min with 0.1% (v/v) TFA for 5 min initially and then with a combination of linear gradients which were 0—19% (v/v) MeCN in 0.1% (v/v) TFA over 21 min, 19—55% (v/v) over 64 min and 65—85% (v/v) over 40 min.

The absorbances at 210 and 215 nm were used to detect proteins and peptides. Each peak fraction was collected, MeCN was removed by a N_2 stream and the resultant was lyophilized for further analyses.

Enzymatic Digestion Samples, 500 μg, were dissolved in 0.5 ml of 4 M urea/50 mM Tris–HCl (pH 8.5) containing 20 μg *Achromobacter* protease I. The solutions were incubated for 24 h at 37 °C, and then the resultants were reduced with 3% 2-mercaptoethanol for 2 h at 37 °C.

Chemical Analysis Samples, 3—20 nmol, were hydrolyzed for 24 h at 110 °C in 0.5 ml of 6 N HCl containing 4% (v/v) thioglycolic acid *in vacuo*. Cysteine was oxidized to cysteinic acid according to the method of Moore[11] prior to hydrolysis. Amino acid analysis was carried out with a Hitachi amino acid analyzer, model 835-50 (Hitachi Ltd.). N-Terminal amino acid sequence determination of peptides (1.5—2.2 nmol) was performed with a gas-phase sequencer, model 744A (Applied Biosystems Inc.), by means of automated Edman degradation.

Gel Electrophoresis and Isoelectric Focusing (IEF) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli[12] using 13.5% (w/v) acrylamide gels on a Protean dual slab cell (Bio-Rad Laboratories). Samples, 5 μg, were dissolved in 10 μl of 0.1 M Tirs–HCl (pH 6.8) containing 2% (v/v) SDS, and if necessary, reduced with 2% (v/v) 2-mercaptoethanol for 1 h at room temperature. After electrophoresis, proteins were stained with 0.1% (w/v) Coomassie brilliant blue R-250.

IEF was performed with an Ampholine PAG plate (pH 3.5—9.5) (Pharmacia LKB Biotechnology). Samples, 10 μg, were loaded onto a gel using filter paper, and 1 M sodium hydroxide and 1 M phosphoric acid were used as the cathode and anode electrolytes, respectively. IEF was carried out at a constant power of 1.3 W/cm for 2 h at 4 °C on an isoelectric focusing apparatus equipped with horizontal electrophoresis cells, model 1415 and 1405 (Bio-Rad Laboratories). After IEF, proteins were stained as in the case of SDS–PAGE.

Other Procedure The biological activity of rIL-2 was determined according to the method reported by Tada *et al.*[13] Protein was determined by the Lowry's method[14] with bovine serum albumin as the standard.

Results

Chromatographic Behavior of rIL-2 Kept in Aqueous Solutions rIL-2 was hardly modified immediately after purification. However, when rIL-2 was kept in aqueous solutions at 25 °C, it was gradually modified first to the oxidized form[8] in which Met^{104} is oxidized to methionine sulfoxide. Upon storage for periods exceeding 2 months, an additional component (D-1) appeared, and it was produced to the extent of 2% upon storage for 6 months, as is shown in Fig. 1. The pH and the temperature dependence of the rate of production of D-1 were investi-

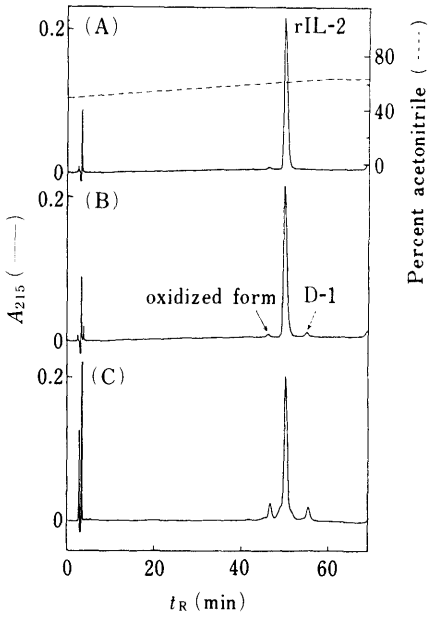

Fig. 1. Chromatographic Behavior of rIL-2 Kept in Aqueous Solutions

(A) rIL-2 final preparation, (B) 6 months at 25 °C, (C) 2 months at 40 °C. The applied gradient is shown only on the top chromatogram.

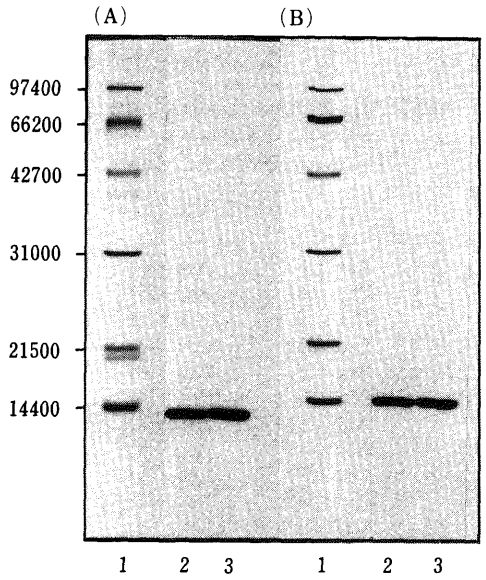

Fig. 2. SDS–Polyacrylamide Gel Electrophoretogram of rIL-2 and D-1

Samples were: lane 1, marker proteins (1 μg each) [lysozyme (molecular weight 14400), soybean trypsin inhibitor (21500), carbonic anhydrase (31000), ovalbumin (42700), bovine serum albumin (66200) and phosphorylase B (97400)]; lane 2, rIL-2 (10 μg) and lane 3, D-1 (10 μg). (A) Non-reducing conditions, (B) reducing conditions.

TABLE I. Specific Biological Activity of rIL-2 and D-1

Sample	Activity[a] ($\times 10^7$ JRU/mg protein)
rIL-2	1.1±0.1
D-1	0.9±0.1

a) Mean ± standard deviation of 3 independent experiments.

gated. Under acidic conditions, pH 3—4, the production rate was almost the same as that at pH 5. Under alkaline conditions, oligomerization and disulfide scrambling took place, and the production rate didn't increase. D-1 production increased with an increase in temperature, and D-1 was produced to the extent of 8% upon storage at

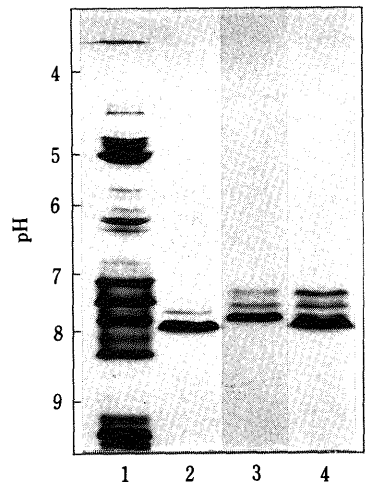

Fig. 3. Gel IEF of rIL-2 and D-1

Samples were: lane 1, marker proteins (10 μg each) [β-lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), house myoglobin-acidic band (6.85), house myoglobin-basic band (7.35), lentil lectin-acidic band (8.15), lentil lectin-middle band (8.45), lentil lectin-basic band (8.65), trypsinogen (9.30) and cytochrome c (10.25)]; lane 2, rIL-2 (10 μg); lane 3, D-1 (10 μg) and lane 4, rIL-2 kept for 2 months at 40 °C.

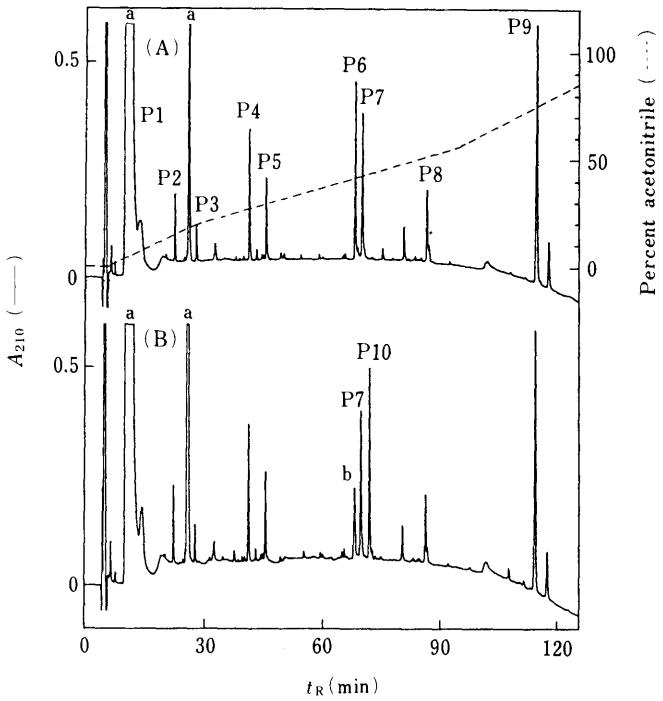

Fig. 4. RP-HPLC of *Achromobacter* Protease I-Digested Peptides of rIL-2 and D-1

(A) rIL-2, (B) D-1. Peak a and peak b were derived from 2-mercaptoethanol and a plastic bottle, respectively. The applied gradient is shown only on the top chromatogram.

40 °C for 2 months, whereas production didn't increase at temperatures above 60 °C because oligomerization occurred. No D-1 was produced at temperatures below 5 °C.

Electrophoretic Properties and Biological Activity The molecular weight of rIL-2 and D-1 was determined by SDS–PAGE (Fig. 2). Both species migrated as single bands with a molecular weight of about 14000 under non-reducing conditions and 15000 under reducing conditions. Their biological activities were almost the same in terms of ability to stimulate the growth of an IL-2-dependent murine cell line (Table I). Both species were subjected to

TABLE II. Amino Acid Composition of *Achromobacter* Protease I-Digested Peptides of rIL-2 and D-1

Amino acid	Number of residues per molecule[a]									
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Asx	1.1 (1)					4.1 (4)	1.1 (1)	4.2 (4)	2.1 (2)	3.6 (4)
Thr		1.7 (2)	0.9 (1)		1.8 (2)			0.9 (1)	6.3 (7)	
Ser		1.9 (3)				0.8 (1)	0.7 (1)		1.7 (3)	0.7 (1)
Glx			1.0 (1)			1.0 (1)	7.4 (7)	4.1 (4)	5.2 (5)	1.0 (1)
Pro	1.0 (1)	1.0 (1)		1.0 (1)		1.0 (1)	0.8 (1)			1.1 (1)
Gly								1.1 (1)	1.0 (1)	
Ala		1.0 (1)	1.0 (1)				1.0 (1)		2.0 (2)	
Cys[b]							1.0 (1)		1.9 (2)	
Val						1.6 (2)	1.1 (1)		1.0 (1)	1.1 (2)
Met				0.9 (1)	0.9 (1)			0.9 (1)	0.9 (1)	
Ile						2.5 (3)		1.9 (2)	3.7 (4)	1.8 (3)
Leu			1.0 (1)		2.1 (2)	4.4 (4)	6.8 (6)	7.5 (7)	2.0 (2)	3.4 (4)
Tyr				1.0 (1)				1.0 (1)	1.0 (1)	
Phe				1.0 (1)	1.0 (1)	1.1 (1)			3.1 (3)	0.9 (1)
Lys	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	2.0 (2)	1.0 (1)		1.0 (1)
His						1.0 (1)	1.0 (1)	1.0 (1)		1.0 (1)
Arg					1.0 (1)	2.0 (2)			1.0 (1)	2.0 (2)
Trp									0.5 (1)	
Yield (%)	59.4	63.4	67.2	64.4	14.8	54.4	30.9	26.6	47.0	51.2
Peptide assignment	Asn^{33} | Lys^{35}	Ala^{1} | Lys^{8}	Ala^{50} | Lys^{54}	Phe^{44} | Lys^{48}	Leu^{36} | Lys^{43}	Asn^{77} | Lys^{97}	His^{55} | Lys^{76}	Thr^{10} | Lys^{32}	Gly^{98} | Thr^{133}	Asn^{77} | Lys^{97}

a) The values of Lys or Leu were taken as standards. The numbers in parentheses are the number of amino acids predicted from the cDNA sequence of rIL-2. *b*) Cys was detected as Cysteic acid ($Cys(SO_3H)$) by oxidation with performic acid.

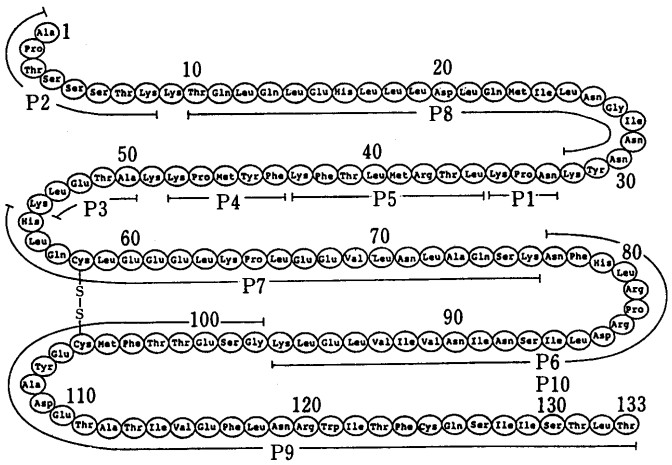

Fig. 5. Amino Acid Sequence of rIL-2 Predicted from the cDNA Sequence of rIL-2

P1—P10 indicate the *Achromobacter* protease I-digested peptides described in Fig. 4.

TABLE III. Amino Acid Sequence Analysis of P6 and P10

Cycle	PTH-amino acid detected (yield %)		Amino acid predicted from the cDNA sequence
	P6	P10	
1	Asn (28)	Asn (30)	Asn
2	Phe (66)	Phe (53)	Phe
3	His (9)	His (8)	His
4	Leu (55)	Leu (43)	Leu
5	Arg (11)	Arg (10)	Arg
6	Pro (27)	Pro (24)	Pro
7	Arg (6)	Arg (8)	Arg
8	Asp (21)	Asp (9)	Asp
9	Leu (29)	Leu (20)	Leu
10	Ile (24)	Ile (17)	Ile
11	Ser (2)	Ser (2)	Ser
12	Asn (14)	Asp (8)	Asn
13	Ile (16)	Ile (10)	Ile
14	Asn (13)	Asn (8)	Asn
15	Val (12)	Val (6)	Val
16	Ile (11)	Ile (7)	Ile
17	Val (11)	Val (6)	Val
18	Leu (9)	Leu (5)	Leu
19	Glu (3)	Glu (6)	Glu
20	Leu (7)	Leu (5)	Leu
21	Lys (2)	Lys (1)	Lys

PTH, phenylthiohydantoin.

IEF. rIL-2 and D-1 focused mainly at pI 7.9 and 7.6, respectively (Fig. 3). rIL-2 which had been kept at 40 °C for 2 months was also subjected to IEF. Several species other than rIL-2 and D-1 were observed, suggesting that additional degradation reactions occur under these storage conditions (Fig. 3).

Peptide Mapping rIL-2 and D-1 were digested with *Achromobacter* protease I followed by reduction, and each resulting peptide mixture was subjected to RP-HPLC. Since both species which were fractionated by RP-HPLC have poor solubility, they were digested in the presence of a denaturant, 4 M urea. As shown in Fig. 4, rIL-2 gave nine major peptides, P1—P9. P1 was obtained from a non-reduced digest, since it was co-eluted with the reducing agent, 2-mercaptoethanol. Amino acid analysis showed that all these peptides corresponded to the peptides predicted from the complementary DNA (cDNA) sequence of rIL-2, except for the liberation of Lys^{9} and Lys^{49} (Table II, Fig. 5). In contrast, D-1 did not give P6 which was obtained from rIL-2 but alternatively gave a new peptide, P10. All other peptides were the same as those from rIL-2. An unknown component (peak b) with almost the same retention time as P6 was subjected to amino acid analysis. No amino acids were detected

indicating that the unknown component was not another new peptide but came from the plastic bottle used in the fractionation. As shown in Table II, the amino acid composition of P10 is the same as that of P6 considering that highly hydrophobic sequences often give hydrophobic amino acid yields lower than the theoretical values upon hydrolysis. These results suggest that some modification took place in the region corresponding to Asn^{77}–Lys^{97} in rIL-2. Since amido group-containing amino acids such as asparagine and glutamine are hydrolyzed to aspartic acid and glutamic acid, respectively, during acid hydrolysis on amino acid analysis, it seems that the difference between P6 and P10 is due to the modification of amido groups. To clarify this, both peptides were subjected to N-terminal amino acid sequence analysis. As shown in Table III, P10 is deamidated at position 12. Consequently, it was confirmed that D-1 is a species in which only Asn^{88} is deamidated.

Discussion

Deamidation of asparagine and glutamine residues in proteins is sometimes encountered upon purification and storage, and hence the effect of temperature and pH on deamidation has been thoroughly investigated in insulin,[15] cytochrome c[16] and recombinant interleukin 1α.[17] Fisher and Porter[15] have shown that deamidation and oligomerization of bovine insulin occur slowly even in crystalline powder. In the case of rIL-2, however, deamidation was observed when it was kept in 5 mM ammonium acetate buffer solution (pH 5.0) at 25 and 40 °C for periods exceeding 2 months. When rIL-2 was kept at higher temperatures or in aqueous solutions of other pH, oligomerization or disulfide scrambling occurred predominantly producing only a small amount of D-1. Consequently, it seems that the mode of degradation, such as deamidation, oligomerization and disulfide scrambling, varies with pH, since varying the surrounding environment causes a change in the dissociative state and an accompanying conformational change in the rIL-2 molecule.

Daumy *et al.*[18] have reported that murine recombinant interleukin 1β is easily deamidated at asparagine-149, and the deamidated form has 3- to 5-fold lower biological activity than the unmodified form. Despite the deamidation, there seems to be little structural change around the active site, as D-1 has almost the same biological activity as rIL-2. Whereas the pI value of rIL-2, 7.9, agrees very well with the theoretical value estimated from the p*K* values of all dissociative groups in the molecule, D-1 has a pI value, 7.6, which differs from the theoretical value, 6.4. Hydrophobic amino acid residues such as valine, isoleucine and leucine are localized in the vicinity of the deamidated Asn^{88}, and the region including Asn^{88}, Leu^{85}–Ile^{92}, is estimated to be highly hydrophobic using the hydrophilic parameters proposed by Hopp and Wood.[19] Consequently, it is assumed that it is difficult for the carboxyl group of Asp^{88} in the hydrophobic region to contribute to the charge distribution of D-1 resulting in a pI value higher than the theoretical value.

Brandhuber *et al.*[20] have predicted by means of X-ray crystal structure analysis that the region Arg^{83}–Thr^{101} is located on the exterior of the molecule having an α-helical structure, and a few asparagine and glutamine residues also exist in other external regions. Amido groups other than Asn^{88} might be partially deamidated, because several unknown species in addition to D-1 were observed on IEF, as shown in Fig. 3. Therefore, the mixture of these unknown species was fractionated by chromatofocusing and digested with *Achromobacter* protease I, and then peptide mapping of the resulting digest was performed. The amount of peptide P2 was consequently decreased, and a few new peptides appeared, whereas all other peptides were the same as those from rIL-2. These new peptides were subjected to amino acid analysis. The elimination of the N-terminal amino acid occurred, but the deamidation of asparagine and glutamine residues did not. This suggests that it is difficult for the deamidation reaction to take place at positions other than Asn^{88} although rIL-2 has eight asparagine residues (positions 26, 29, 30, 33, 77, 88, 90 and 119) and six glutamine residues (positions 11, 13, 22, 57, 74 and 126). It is not clear why Asn^{88} in the hydrophobic region is specifically deamidated, when several asparagine and glutamine residues are assumed to exist on the exterior of the protein molecule and should likely be deamidated as well.

Prior studies[21] have suggested that the intermediate formation of a five-membered succinimide ring may contribute to deamidation at asparagine residues in proteins and peptides, and isoaspartic acid may be produced by isomerization. If a peptide has an isoaspartic acid residue, Edman degradation is known to stop at that position on sequence analysis. Edman degradation of P10, however, did not stop at position 88. This suggests that isomerization does not occur upon the deamidation of rIL-2.

In view of long-term stability, rIL-2 should be kept at temperatures below 5 °C so that deamidation and elimination of the N-terminal amino acid do not occur.

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