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Reduction of biological activity of murine recombinant interleukin-1β by selective deamidation at asparagine-149

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A biologically active preparation of murine recombinant interleukin-1β (mIL-1β) from *Escherichia coli* cell lysates contained two forms of mIL-1β with pI 8.7 and pI 8.1, respectively. Treatment with 0.1 M Tris, pH 8.5, at 37°C for 35 h converted the pI 8.7 form to the pI 8.1 form by the selective deamidation of an asparagine residue (Asn^{149}) in the mIL-1β molecule. Deamidated mIL-1β had 3- to 5-fold lower co-mitogenic activity and receptor affinity than the unmodified form.

Interleukin-1; Cytokine; Deamidation

1. INTRODUCTION

IL-1[1] mediates a range of biological activities including the activation of cells involved in immune and inflammatory responses [1,2]. The two molecular forms of mature (17 kDa) human IL-1, α (pI~5.0) and β (pI~7.0), are only about 25% homologous in sequence but bind to the same receptor [3] and share elements of 3D structure [4–6]. Although most attention has centered on the human proteins, mIL-1α and mIL-1β are valuable research reagents that have been produced by recombinant expression in *E. coli* [7–9]. This report describes a modification of the structure of mIL-1β that was noted during its recovery from bacterial lysates and caused heterogeneity in the isolated recombinant product. The heterogeneity was due to selective deamidation of a single Asn residue, and the results are of interest both in terms of structure–activity relationships of IL-1β and with regard to the structural factors governing deamidation of amide residues in proteins.

2. MATERIALS AND METHODS

2.1. *Protein purification*

mIL-1β was precipitated from bacterial cell lysates with ammonium sulfate and recovered from the 25–80% saturated fraction by centrifugation. The protein was dissolved in buffer (50 mM Tris, 1 mM EDTA, 1 mM DTT, 1 mM NaN_3, pH 7.0), desalted on a Sephadex G-25 column, and fractionated on a DE-52 cellulose column equilibrated in the same buffer.

2.2. *Analytical biochemical methods*

The procedures used for SDS-PAGE, IEF, cation exchange chromatography, molecular exclusion HPLC and amino acid analysis have been described [9]. Protein sequencing was performed using an Applied Biosystems Model 470A gas-phase sequencer equipped with a Model 120A on-line PTH analyzer. Alternatively, PTH amino acids were identified in off-line mode using a Zorbax PTH column (DuPont). Data were acquired, stored and analyzed using a PE Nelson data system.

3. RESULTS

3.1. *Expression, purification and activity of mIL-1β*

To obtain mIL-1β, a semisynthetic gene encoding an initiator Met followed by the C-terminal 152 amino acids (residues 118–269) of the mIL-1β precursor [10] was expressed in *E. coli* as 10–15% of the total cellular protein. The recombinant cytokine, which was recovered exclusively from the soluble fraction of cell lysates, was isolated and purified by ammonium sulfate fractionation and anion exchange chromatography. The isolation procedure yielded a product which appeared homogeneous by SDS-PAGE (Fig. 1A) but was resolved by IEF into two protein species with pIs of 8.7 and 8.1 (Fig. 1B).

The two mIL-1β species were isolated by cation exchange chromatography (Fig. 1C) and each was analyzed separately. The proteins exhibited identical amino acid compositions and N-terminal sequences (10 residues), each retaining the initiator methionine. They also had the same molar absorptivity (15000 $M^{-1} \cdot cm^{-1}$ at 280 nm). They had identical M_r as judged by molecular exclusion HPLC and SDS-PAGE, and both

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Abbreviations: IL-1, interleukin-1; mIL-1, murine IL-1; hIL-1, human IL-1; IEF, isoelectric focusing; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; GnHCl, guanidine hydrochloride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; PTH, phenylthiohydantoin

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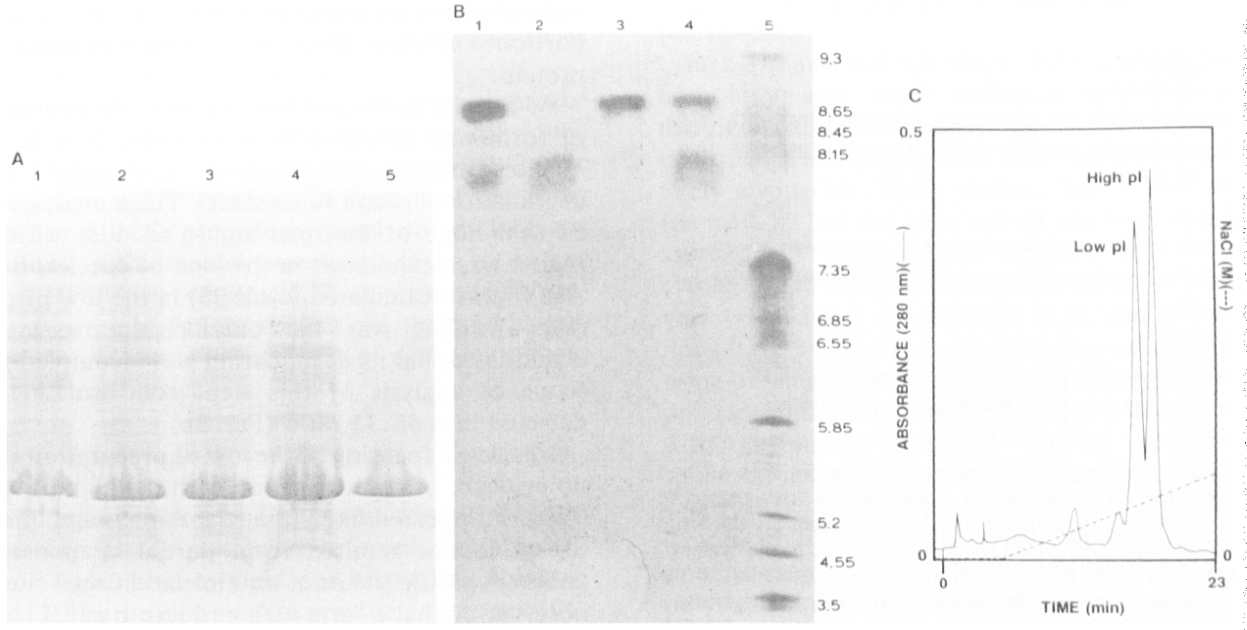

Fig. 1. (A) SDS polyacrylamide gel electrophoresis showing isolation of recombinant mIL-1β. Lane 1, total cell lysate; lane 2, soluble fraction of lysate; lane 3, 25–80% saturated ammonium sulfate fraction; lane 4, desalted pooled protein fraction; lane 5, DEAE-52 pooled fractions (0.47 μg loaded). (B) Isoelectric focussing of mIL-1β after incubation in alkaline buffer. Lanes 1 and 4, mIL-1β before MonoS column; lane 2, first MonoS peak (pI 8.1) (see panel C); lane 3, second MonoS peak (pI 8.7); lane 5, IEF standards. (C) Cation exchange chromatography. mIL-1β high and low pI species were isolated on a Pharmacia MonoS HR 10/10 cation exchange column using a NaCl gradient in 0.1 M NH_4HCO_2, pH 5.3.

proteins contained a cysteinyl residue which was detected with DTNB only after denaturation with 6 M GnHCl.

In the thymocyte co-mitogenic assay [11], the pI 8.1 species was consistently less active than the pI 8.7 species. The average specific activity from 6 determinations with 3 separate preparations of each form of the cytokine were $2.7 \pm 1.3 \times 10^8$ U/mg for the high pI species and $0.6 \pm 0.13 \times 10^8$ U/mg for the low pI form with a mean 5.2 ± 1.4-fold decrease in activity as determined from 6 paired comparisons. The low pI form also exhibited a 3-fold decrease in affinity for the IL-1 receptor. In a competitive receptor binding assay with 28 pM [^{125}I]hIL-1α [11] the average effective concentration causing 50% displacement by the high and low pI mIL-1β species were 133 ± 14 pM and 356 ± 50 pM, respectively.

3.2. *Formation of the less active form of mIL-1β*

The pI 8.7 protein (0.9 mg/ml) was converted to the less biologically active pI 8.1 species by incubation at 37°C in 0.1 M Tris, pH 8.5. The rate of conversion was determined by cation exchange chromatography, and a reaction half-life of 35 h was calculated. IEF analysis of reaction mixture aliquots demonstrated the appearance of a diffuse band at pI 8.1 with the concomitant disappearance of the pI 8.7 band. However, upon prolonged incubation, the intensity of this pI 8.1 band also decreased with the concomitant appearance of a third protein band at pI 7.0.

While this observation tended to support the hypothesis that deamidation of Asn and/or Gln residue(s) caused the formation of the low pI species, the alkaline conditions used in these experiments were too mild to effect indiscriminate protein deamidation [12–14]. Selective deamidation of one or more residues appeared more plausible and the task of identifying the site(s) of deamidation was undertaken.

3.3. *Structural analysis of the less active form of mIL-1β*

The primary structure of mIL-1β includes 9 Asn and 11 Gln residues (Fig. 2). The initial strategy followed to identify deamidation at any of these sites was to digest each protein with *Staphylococcus aureus* V8 protease. In phosphate buffer, *S. aureus* V8 protease cleaves proteins at the carboxyl side of both aspartyl and glutamyl residues [15]. Therefore, digestion of the two mIL-1β species might have generated different peptide maps because deamidation would have produced new peptide cleavage sites. Comparison of the HPLC peptide maps (not shown) failed to reveal any difference between the two forms, but the peptides recovered in this analysis accounted for only 57% of the total protein. From these maps the first 13 N-terminal residues (residues 118–130) and the last 73 C-terminal residues (residues

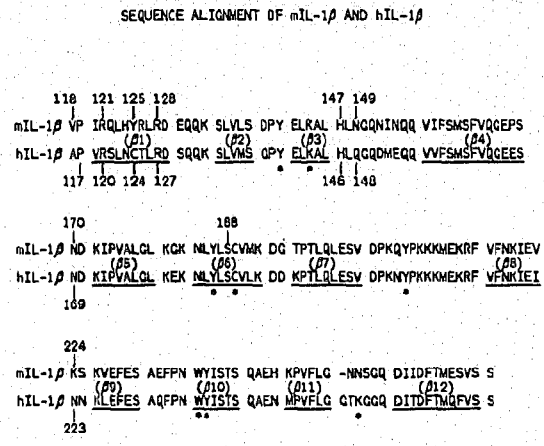

Fig. 2. Amino acid sequence alignments of mature mIL-1β and hIL-1β. Residues were numbered according to the respective precursor molecules. Portions of the hIL-1β sequence which are underlined below the amino acid residues correspond to the twelve β-strands (β1–β12) [5,6]. Residues not underlined correspond to the connectors between the strands. Residues which upon substitution result in neutral mutations [21–24] are underlined by an asterisk (*). The initiator Met present in recombinant-derived mIL-1β is not shown.

193–269) were identified; since the maps were identical, selective deamidation of any Asn or Gln among these residues was ruled out. However, the portion of the protein (43%) encompassing residues 131–196 (Fig. 2) was not recovered from the HPLC experiment, and this portion contained 11 of the 20 amide residues in the protein.

As a direct analysis of this region of the protein, each pI form was subjected to an extended N-terminal sequence analysis (continuous automated Edman degradation through 40 residues). These analyses showed that none of the nine amide residues within this region was deamidated in the high pI species, but that Asn^{149} was deamidated (cycle 33) in the low pI protein (Fig. 3). This was the only residue seen to be deamidated, but its deamidation was incomplete; in the sequence analysis of this step, Asp and Asn were detected in a 65:35 ratio (Fig. 3b).

Isoelectric focusing of the low pI preparation showed no evidence of contamination with the high pI species. Thus, it appeared likely that the remaining 35% of pI 8.1 molecules resulted from partial deamidation at another specific site or at multiple additional sites. The observation that a form with a pI less than 8.1 could be generated during the alkaline incubation (see above) supported the contention that limited deamidation occurred at additional sites.

CNBr digestion followed by covalent chromatography on thiopropyl Sepharose (not shown) was used to isolate the Cys^{188} containing peptide encompassing residues 162–191; this peptide contained the three amide residues (Gln^{165}, Asn^{170} and Asn^{183}) unac-

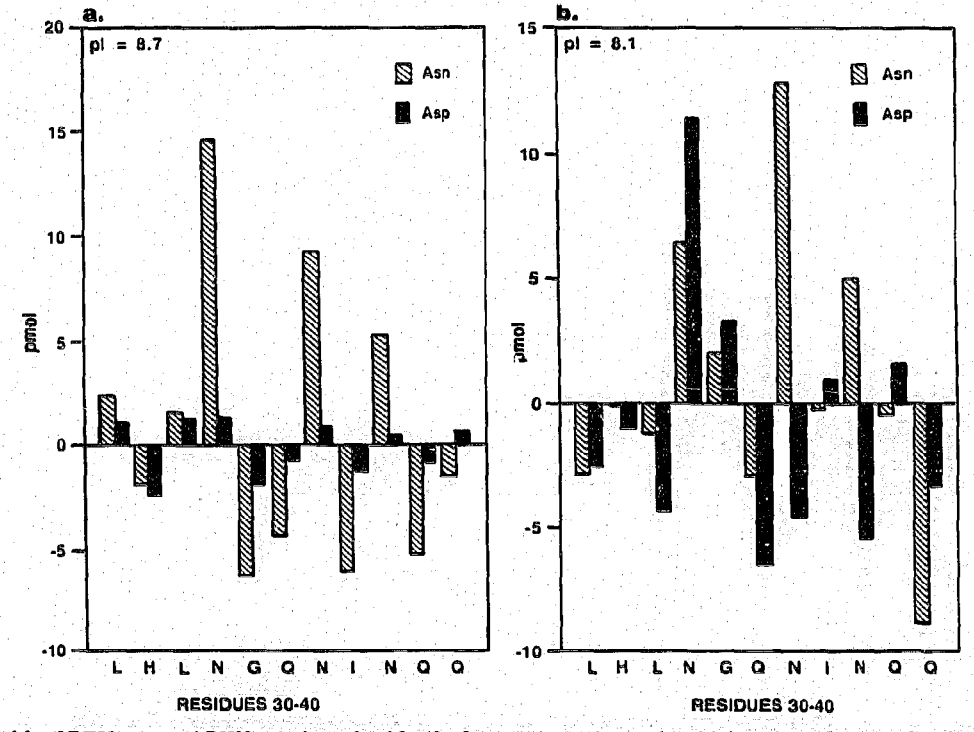

Fig. 3. Changes in yields of PTH-Asn and PTH-Asp in cycles 30–40 of sequence analyses of (a) high and (b) low pI mIL-1β. Yields are uncorrected for lag and background variations.

counted for in the analysis. Sequencing of each Cys^{188} containing fragment from both high and low pI species failed to reveal any deamidation.

Because of the critical nature of the experiment, the extended N-terminal sequencing of the two pI forms was performed twice using different methods. On the first occasion, PTH amino acids were analyzed using an off-line HPLC procedure based on an isocratic separation. In the second experiment (Fig. 3), on-line gradient HPLC was employed. Data from the two experiments were in full agreement, so that the assignment of Asn^{149} as the primary site of deamidation was made with confidence.

4. DISCUSSION

Deamidation of Asn or Gln residues in polypeptides occurs through formation of a cyclic intermediate which is hydrolyzed to yield one of two possible products [12]. In the case of Asn residues, these products are (i) a normal peptide in which the native Asn has been replaced by Asp or (ii) a peptide isomer in which Asn has been replaced by an isoaspartyl residue [12]. In either case, a carboxyl group is present in the residue that replaces the original neutral Asn and the modified protein has a lower pI than the native protein. Sequence and local conformation are both factors in governing the susceptibility of any individual Asn to deamidation [16].

Automated Edman sequencing revealed that the exceptional lability to deamidation of bacterially expressed mIL-1β was mainly due to the ease with which this modification occurred at Asn^{149} (residue 33 of the methionyl mature cytokine). The pI 8.1 form of the molecule was at least 65% deamidated to Asp at this position, while a further fraction of Asn^{149} may have been converted to an isoaspartyl residue which could not have been detected by the methods applied. Edman sequencing is arrested in chains where the latter event has occurred [17], so that the estimate of 65:35 distribution between Asp and Asn for residue 149 of the pI 8.1 form does not take into account the possible presence of molecules in which Asn^{149} has been converted to isoaspartic acid. The fact that sequencing continued past the deamidation site without major loss of yield is consistent with the interpretation that conversion to Asp was the principal product of the alkali-induced chemical modification in the present case. This result contrasted with that observed with human growth hormone in which isoaspartyl was by far (70–80%) the major product of deamidation of an Asn residue [18].

Because Asn^{149} is located within the only Asn-Gly sequence of mIL-1β, precedent identified it as the most likely candidate for enhanced susceptibility to deamidation [19,20]. Formation of the cyclic imide intermediate involved in the reaction is favored by the presence of a small residue (especially Gly) immediately after Asn [12,18–20].

mIL-1β and hIL-1β share 84% primary sequence homology and their biological activities are indistinguishable. Because the two proteins are so similar in sequence and in function, their tertiary structures are likely also to be closely similar. The tertiary structure for mIL-1β has not been solved nor have site-directed mutagenesis studies been reported for the murine cytokine. However, the crystal structure of hIL-1β has been refined to 2 Å resolution [5,6], and several mutations have been introduced into this molecule (Fig. 2), some of which negatively affect cytokine function. The point mutations shown to produce hIL-1β molecules with decreased biological activity correspond to substitutions at the following residues: Arg^{120} [23], Cys^{124} [21], Arg^{127} [24] and His^{146} [22]. Upon sequence alignment of the two proteins, these residues correspond to Arg^{121}, Tyr^{125}, Arg^{128} and His^{147} in the murine polypeptide (Fig. 2). In the hIL-1β structure, Cys^{124} is inaccessible to solvent whereas Arg^{120} and Arg^{127} are exposed [5,6]. While these 3 residues are located within the first β-sheet of the hIL-1β molecule, His^{146} forms part of the connecting loop between β-sheets 3 and 4 [5,6]. Clearly, mutations which affect binding to the receptor, for example at His^{146}, must affect biological activity because internalization of IL-1β is required for expression of its activity [25,26]. Loss of cytokine function, however, does not necessarily imply loss of receptor binding. In fact, receptor binding and biological activity have been uncoupled in an R127G hIL-1β mutant protein [24]. This mutation results in a biologically inactive molecule which essentially retains its ability to bind to the receptor [24]. Our results show that introduction of a negative charge at Asn^{149} resulted in a murine cytokine with significantly reduced receptor binding affinity and thymocyte co-mitogenic activity. The homologous residue to Asn^{149} in the human species is Gln^{148} which, like His^{146}, is exposed to solvent and forms part of the connector between β-sheets 3 and 4 [5,6]. This connecting loop in hIL-1β includes three sequential type III β-turns, forming a 3_{10}-helical structure encompassing residues Gly^{149} through Gly^{154} [5]. Based on our observations with mIL-1β deamidated at Asn^{149} and observations of others on hIL-1β [21–24] we suggest that, in addition to β-sheet 1, the loop connecting β-strand 3 and β-strand 4 may be required to preserve the biological activity of IL-1β and that this loop could represent an important site for receptor binding to the cytokine.

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