

## Recombinant-derived interleukin-1 $\alpha$ stabilized against specific deamidation

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**Recombinant-derived human interleukin-1 $\alpha$  (IL-1 $\alpha$ ), purified from *Escherichia coli*, was resolved by isoelectric focusing on polyacrylamide gels into two species of isoelectric points (pI) 5.45 and 5.20, which constituted ~75% and ~25% of the total IL-1 $\alpha$  protein respectively. The pI 5.45 and pI 5.20 species were separated by chromatofocusing and subjected to N-terminal sequence analysis. The pI 5.45 species contained the expected Asn residue at position 36 of the mature protein sequence whereas the pI 5.20 species contained an Asp residue at the same position. A mutant protein in which Asn-36 was substituted for a Ser residue was isolated from *E. coli* and shown to be homogeneous on isoelectric focusing analysis with a pI = 5.45. <sup>1</sup>H-n.m.r. and circular dichroism analyses of wild-type and the mutant IL-1 $\alpha$  indicated a similar conformation which was also indicated by the identical receptor binding affinities of IL-1 $\alpha$  with Asn, Asp or Ser in position 36. The mutant protein was stabilized against specific base-catalysed and temperature-induced deamidation, and may be more suitable than the wild-type position for physical and structural studies.**

**Key words:** interleukin-1 $\alpha$ /sequence determination/deamidation/site-specific mutagenesis/protein conformation

### Introduction

We have recently described the purification of recombinant human interleukin-1 $\alpha$  (IL-1 $\alpha$ ) from *Escherichia coli* using relatively mild conditions which avoided denaturing solvents and extremes of pH and temperature (Wingfield *et al.*, 1987a). Nevertheless, the preparation was heterogeneous by isoelectric focusing. Two distinct IL-1 $\alpha$  species of pI 5.45 (70–75% of protein) and pI 5.20 (25–30% of protein) were observed. Titration curve analysis on polyacrylamide gel (performed as described by Wingfield *et al.*, 1987b) indicated that the pI 5.20 species was a once-deamidated protein. This observation was of particular interest since Cameron *et al.* (1986) had reported the purification of two species of authentic human IL-1 $\alpha$  from mononuclear cell cultures with similar isoelectric points as the *E. coli*-derived protein. Furthermore, these authors demonstrated that residue 36 of the pI 5.20 species was an Asp, whereas the pI 5.45 species contained an Asn in the same position (note residue 36 of the mature protein sequence is equivalent to residue 148 of the precursor sequence listed by March *et al.*, 1985). They suggested that this sequence difference might account for the small charge

difference between the two species and may be due to either a single base change or deamidation. Both authentic IL-1 $\alpha$  species were shown to have similar biological activities.

In this report, by direct N-terminal sequencing of the *E. coli*-derived IL-1 $\alpha$  species, we confirm the presence of Asn-36 in the pI 5.45 species and Asp-36 in the pI 5.20 species. Using site-specific mutagenesis to substitute the Asn-36 for a serine residue, we show that this mutant IL-1 $\alpha$  is purified from *E. coli* as a single charge species (pI = 5.45) with similar properties to the wild-type protein. The IL-1 $\alpha$  mutant, thus stabilized against specific deamidation, is suggested to be a more suitable starting material for physical characterization studies such as <sup>1</sup>H-n.m.r. and X-ray crystallographic analysis.

The use of site-specific mutagenesis to substitute protein Asn residues susceptible to deamidation was previously reported by Ahern *et al.* (1987) studying the thermostability of yeast triosephosphate isomerase.

### Materials and methods

#### *Preparation of wild-type and mutant IL-1 $\alpha$*

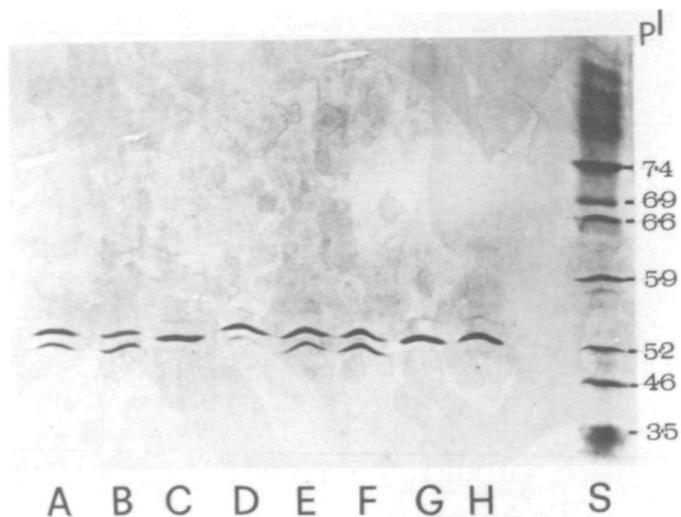
Site-specific mutagenesis was used to introduce the point mutation in the IL-1 $\alpha$  molecule. The template was the phage M13 derivative mp8 carrying the coding region for mature IL-1 $\alpha$ . Mutagenesis was carried out by the double-primer procedure (Norris *et al.*, 1983) with oligonucleotides carrying one or two mismatches. After verification of the mutation by the dideoxy sequencing method (Sanger *et al.*, 1977), the modified IL-1 $\alpha$  gene was transferred to a plasmid to be expressed under control of the  $\lambda$ P<sub>L</sub> promoter and the bacteriophage Mu *ner* gene ribosome binding site. The *E. coli* host was W3110 cI857, carrying the gene for the temperature-sensitive repressor of phage  $\lambda$ . Fermentation and protein purification procedures were as previously described (Wingfield *et al.*, 1987a).

#### *Analytical separation methods*

SDS-PAGE and isoelectric focusing on thin-layer polyacrylamide gels were performed as previously described (Wingfield *et al.*, 1987b).

#### *Chemical analysis*

Prior to analysis, samples were either directly dialysed against 0.1% (w/v) SDS and 2 mM NaCl, or electroeluted from SDS-PAGE gel slices (Hunkapillar *et al.*, 1983) and then dialysed. N-terminal amino acid sequence determination by automated Edman degradation was performed with a gas-phase sequencer (Applied Biosystems Model 470A) using the regular program 03RpTH. In order to minimize the normal increase in cycle lag after Pro residues, the time of the cleavage step for Pro residue 3 was extended by 50%. Identification and quantitation of the phenylthiohydantoin derivatives was performed on line using an Applied Biosystems Model 120A PTH amino acid analyser with external standard quantitation. Amino acid analysis was carried out as previously described (Wingfield *et al.*, 1986).



**Fig. 1.** Isoelectric focusing of wild-type IL-1 $\alpha$  and IL-1 $\alpha$  Asn-36-Ser. Lanes A-H and S refer to isoelectric focusing on thin-layer polyacrylamide gels. Lane S, standard proteins, the pI values are indicated; lanes A and E, wild-type IL-1 $\alpha$ ; lane B, wild-type IL-1 $\alpha$  incubated at pH 10.5 for 3 h at 30°C; lane F, wild-type IL-1 $\alpha$  in 100 mM Tris-HCl, pH 7.5, incubated for 12 h at 42°C, lanes C and G, mutant IL-1 $\alpha$  Asn-36-Ser; lanes D and H as B and F, for IL-1 $\alpha$  Asn-36-Ser. The apparent differences in pI for the same charge species across the gel was due to heating effects.

#### <sup>1</sup>H-n.m.r. spectroscopy

Sample preparation and measurements were made as detailed elsewhere (Gronenborn *et al.*, 1986; MacDonald *et al.*, 1986)

#### Circular dichroism

Circular dichroism spectra were measured using a Jobin-Yon Dichrograph IV linked to a BBC microcomputer for recording data. Spectra are the average of four scans with the base line subtracted. Protein concentrations were estimated using a calculated  $A_{1\text{cm}}^{1\%} = 11.4$  at 280 nm and the mean residue mol. wt was calculated as 113.2. All solutions were filtered (0.22  $\mu\text{m}$ , Millipore GVWP filters) before use.

#### IL-1 $\alpha$ receptor binding assay

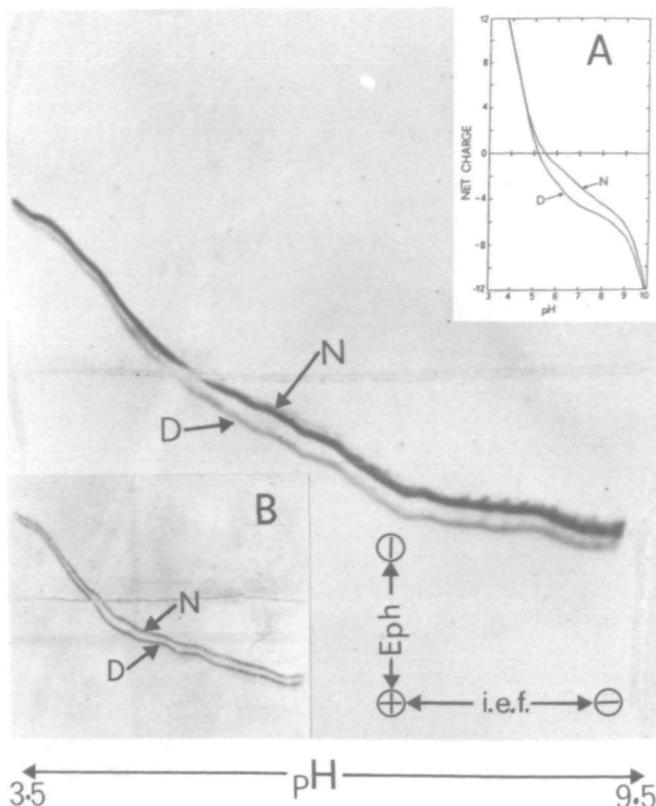
The assay procedure has been described in detail elsewhere (Lowenthal and MacDonald, 1986; MacDonald *et al.*, 1986).

## Results

#### Chemical characterization

Analytical isoelectric focusing of IL-1 $\alpha$  resolved a major and minor species (75% and 25% of the total protein) with respective pI values of 5.45 and 5.20 (Figure 1, lane A). These two species were separated by chromatofocusing as previously described (Wingfield *et al.*, 1987a) and subjected to N-terminal protein sequence analysis. Analysis of the first 40 residues of the IL-1 $\alpha$  pI 5.45 species confirmed the sequence of the mature protein, residues 113-152 of the precursor sequence described by March *et al.* (1985). Analysis of the first 40 residues of IL-1 $\alpha$  pI 5.20 species also confirmed the IL-1 $\alpha$  N-terminal sequence except that residue 36 was an Asp residue rather than the Asn predicted by the cDNA gene sequence. The difference in pI between the pI 5.45 and 5.20 species thus appears to arise as a result of deamidation of Asn-36.

Electrophoretic titration curve analysis is a technique which displays the net charge of a protein as a function of pH. When applied to IL-1 $\alpha$  which had not been subjected to the chromatofocusing separation, two mobility curves (D) and (N)



**Fig. 2.** Titration curve of IL-1 $\alpha$ . Titration curve of IL-1 $\alpha$  in the pH range 3.5-9.5. Conditions for the preparation of the 7.5% (w/v) polyacrylamide gel and running conditions were as described by Wingfield *et al.* (1987b). In the lower right-hand corner the two double arrows with positive and negative symbols indicate the direction and polarity of isoelectric focusing (i.e.f.) and electrophoresis (Eph.). Mobility curves (N) and (D) correspond to non- and once-deamidated IL-1 $\alpha$  respectively. Insert (A) is the theoretical titration curves corresponding to non- and once-deamidated IL-1 $\alpha$ . The curves were generated using a simple calculation procedure based on the Henderson-Hasselbach equation. The  $pK_a$  values of the titratable groups were based on those given by Matthew *et al.* (1978) except for His where an average value of 6.8 was used. Insert (B) is the titration curve analysis of IL-1 $\alpha$  treated with alkali and corresponds to the sample shown in Figure 1, lane B (N) and (D) used in the inserts have the same significance as described above.

were observed (Figure 2) corresponding to the pI 5.20 and pI 5.45 species observed by one-dimensional isoelectric focusing. Theoretical titration curves (Figure 2, insert A) of non- and once-deamidated IL-1 $\alpha$  were similar to the experimentally derived curves supporting the interpretation that the experimentally derived curves (D) and (N) correspond to non- and once-deamidated IL-1 $\alpha$ .

In an attempt to prepare an IL-1 $\alpha$  stabilized against the specific deamidation, we substituted Asn-36 for a Ser residue by site-specific mutagenesis. The mutant protein was purified from *E. coli* and subjected to isoelectric focusing (Figure 1, lane C). Only one major species (97% of the total protein) was observed which had a pI (5.45) identical to the non-deamidated component of wild-type IL-1 $\alpha$ . In addition, a trace amount (3%) of a pI 5.65 species was observed which was also present in the wild-type protein. Polyacrylamide gel electrophoresis in the presence of SDS indicated a single band of 18 000  $M_r$  similar to the wild-type position (result not shown).

The amino acid analysis of the mutant protein (Table I) compared with that of the wild-type protein indicates, as expected, one residue less of Asx/mol protein and a slightly higher Ser con-

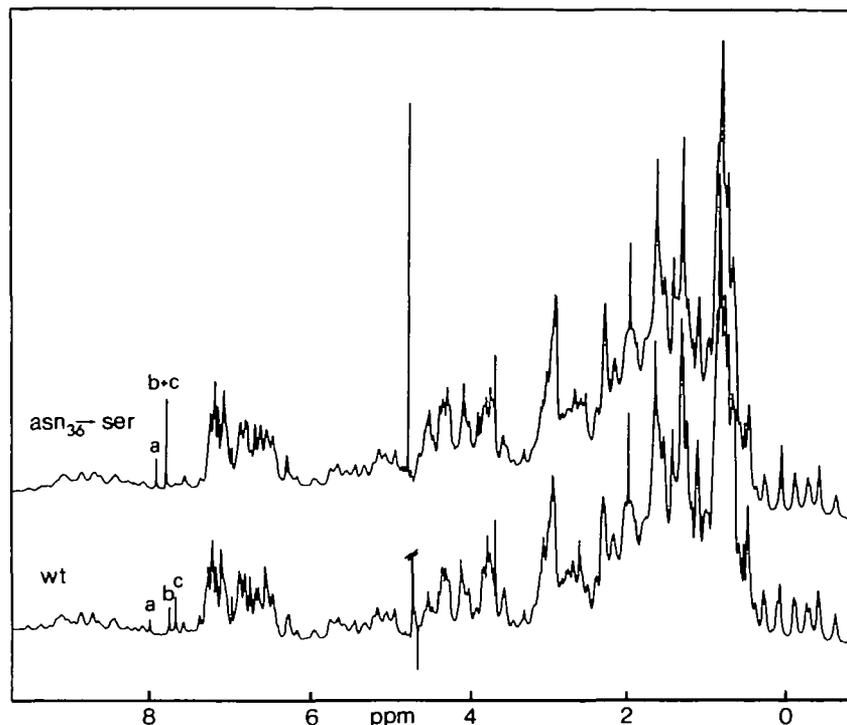


Fig. 3. 500-MHz  $^1\text{H}$ -n.m.r. spectra of wild-type IL-1 $\alpha$  and IL-1 $\alpha$  Asn-36 $\rightarrow$ Ser. Positions of the three histidine resonances (a–c) are indicated. Spectra were recorded at 25°C in sodium phosphate buffer, pH 7.5 (wild-type IL-1 $\alpha$ ) and pH 7.6 (IL-1 $\alpha$  Asn-36 $\rightarrow$ Ser).

Table I. Amino acid analysis of IL-1 $\alpha$  Asn-36 $\rightarrow$ Ser

Amino acid	Composition (mol of amino acid/mol protein)	
	IL-1 $\alpha$	IL-1 $\alpha$ Asn-36 $\rightarrow$ Ser
Asx	19.88 (20)	18.99 (19)
Thr	12.05 (12)	12.12 (12)
Ser	9.39 (10)	10.09 (11)
Glx	16.19 (16)	16.07 (16)
Pro	7.67 (7)	7.73 (7)
Gly	5.79 (5)	6.01 (5)
Ala	14.05 (14)	14.01 (14)
Val	7.51 (7)	7.62 (7)
Met	3.08 (3)	3.17 (3)
Ile	12.74 (13)	12.50 (13)
Leu	15.10 (15)	14.81 (15)
Tyr	6.84 (7)	6.76 (7)
Phe	10.06 (10)	10.19 (10)
His	3.05 (3)	3.05 (3)
Lys	10.88 (11)	10.90 (11)
Arg	3.00 (3)	3.05 (3)

The values given are the averages of three separate analyses of 24-h hydrolysates. Ser, Thr and Ile values were extrapolated from the analysis of 24-, 48- and 72-h hydrolysates. Cysteine and tryptophan contents were not determined. The values in parentheses are those predicted from the respective DNA sequences.

tent (the latter being difficult to quantitate accurately due to destruction during acid hydrolysis). The sequence position of the replacement amino acid was directly confirmed by N-terminal sequence analysis of the first 40 residues; residue 36 was identified as a Ser residue instead of Asn.

#### Stability testing IL-1 $\alpha$ Asn-36 $\rightarrow$ Ser

The mutant IL-1 $\alpha$ , stabilized against deamidation during expression and purification from *E. coli*, was incubated under condi-

tions known to promote deamidation, e.g. acid and alkali pH and elevated temperature (Robinson and Rudd, 1974). After incubation of the wild-type IL-1 $\alpha$  at pH 10.5 for 3 h at 30°C, there was an increase in the pI 5.20 (once-deamidated) species from 25 to 53% of the total protein as shown by isoelectric focusing (Figure 1, lane B). Titration curve analysis (Figure 2, insert B) showed only an increase and corresponding decrease in the protein species (D) and (N) respectively, with no additional species being observed. This confirms that the increased amount of the pI 5.20 species was derived from once-deamidated protein. With the mutant protein under the same conditions there was essentially no change in the amount of the pI 5.45 species (Figure 1, lane D). A trace amount (3–4%) of a pI 5.20 species was observed, but titration curve analysis indicated that this new species was not derived from deamidated protein (result not shown).

A similar analysis as described above was also carried out on the wild-type and mutant protein incubated at pH 3.0, but the results were complicated by protein precipitation under these conditions.

The expression of IL-1 $\alpha$  in *E. coli*, under control of the  $\lambda\text{P}_L$  promoter, is induced at 42°C for 5 h. At 42°C the purified wild-type IL-1 $\alpha$  in 100 mM Tris–HCl, pH 7.5, undergoes deamidation; for example, after 12 h there is an increase from 25 to ~48% in the once-deamidated (pI 5.20) protein (Figure 1, lane F). The mutant protein showed no change in its isoelectric focusing profile after a similar incubation at 42°C, analogous to incubation at alkaline pH (Figure 2, lane H).

#### Conformation and activity

$^1\text{H}$ -n.m.r. spectroscopy was performed to examine possible conformational differences between the wild-type IL-1 $\alpha$  and the mutant protein. Figure 3 shows a comparison of the 500-MHz  $^1\text{H}$ -n.m.r. spectra of IL-1 $\alpha$  and the Asn-36 $\rightarrow$ Ser mutant protein.

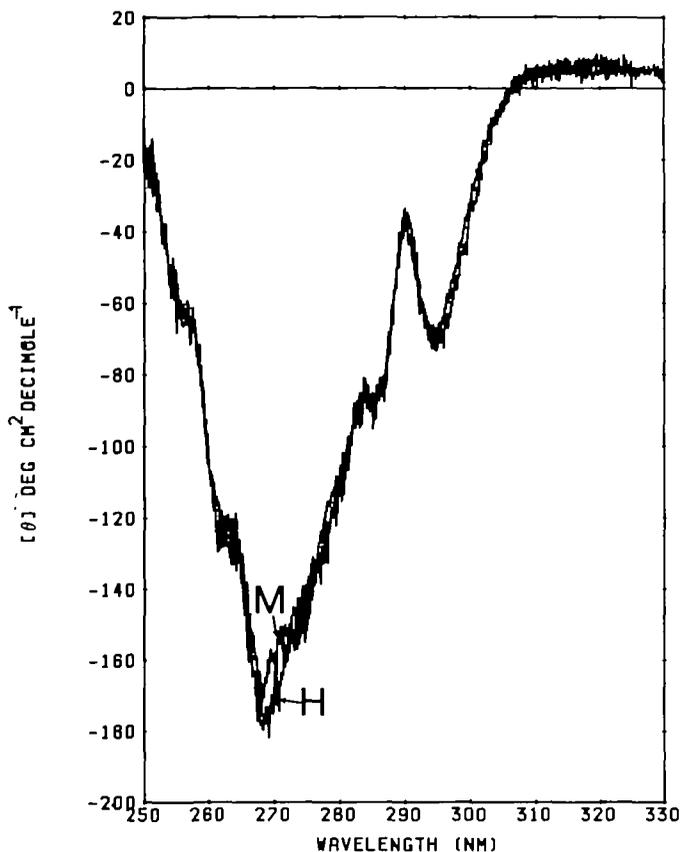


Fig. 4. Near-u.v. circular dichroism spectra of wild-type IL-1 $\alpha$  and IL-1 $\alpha$  Asn-36 $\rightarrow$ Ser. (H) Wild-type IL-1 $\alpha$  (0.78 mg/ml) and (M) mutant IL-1 $\alpha$  (0.65 mg/ml). The buffer was 0.1 M Tris-HCl, pH 7.3. Spectra were recorded at 20°C using a 1-cm pathlength and a 1-nm bandwidth.

Both spectra are very similar, exhibiting only minor changes. This indicates that no gross structural differences exist between the mutant and the native protein. The apparent shift differences for the three histidine  $\epsilon$ 1 proton resonances a, b, c are due to: (i) a slightly different pH in both samples (resonance a) and (ii) to a shift caused by a change in  $pK_a$  for resonances b and c, as evaluated from complete titration curves (data not shown). This  $pK$  change of 0.2 and 0.4 pH units towards higher pH values is clearly due to minor conformational changes influencing these particular histidine resonances. These changes are most likely to be local ones since they do not affect the overall protein structure in a major way. Further evidence for conformational identity was that the near u.v. circular dichroism spectra of the two proteins were almost indistinguishable (Figure 4).

The receptor binding affinities of the mutant protein, the once-deamidated (pI 5.20) and non-deamidated (pI 5.45) wild-type proteins were all very similar (Figure 5). These data indicate that neither deamidation nor substitution of Asn-36 for a Ser residue affects the conformation of the protein receptor binding region. We have previously shown that the biological activity, measured by the lymphocyte-activating factor assay (IL-1/LAF), is the same for the wild-type IL-1 $\alpha$  deamidated and non-deamidated proteins (Wingfield *et al.*, 1987a). The IL-1/LAF activity of the mutant protein was, as expected, similar to the wild-type protein (data not shown).

**Discussion**

IL-1 $\alpha$  and  $\beta$  are polypeptide hormones displaying a wide range of biological activities involved in immune and inflammatory

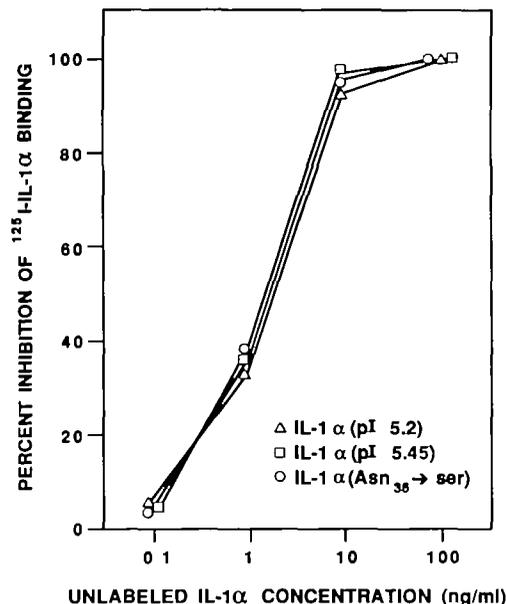


Fig. 5. Competition binding of IL-1 $\alpha$ : comparison of control, deamidated and mutant (Asn-36 $\rightarrow$ Ser) proteins. The indicated concentrations of unlabelled proteins were mixed at 4°C with [<sup>125</sup>I]IL-1 $\alpha$  (1 ng/ml) prior to addition of EL4.6.1. cells ( $5 \times 10^5$ /tube). After 4 h at 4°C, bound radioactivity was evaluated by centrifugation of cells through an oil gradient. Data are expressed as per cent inhibition of [<sup>125</sup>I]IL-1 $\alpha$  binding as compared with untreated controls. Half-maximal binding inhibition was achieved with 1.9 ng/ml IL-1 $\alpha$  (pI 5.2), 1.7 ng/ml IL-1 $\alpha$  (pI 5.45) and 1.6 ng/ml IL-1 $\alpha$  (Asn-36 $\rightarrow$ Ser).



Fig. 6. Amino acid sequence of IL-1 $\alpha$  as predicted from the gene sequence. Sequence positions of the 10 asparagine (N) residues are indicated. The sequence surrounding Asn-36 is underlined.

responses (for a review see Oppenheim *et al.*, 1986). The structural analysis of these proteins, therefore, is important. We have placed special emphasis upon obtaining well-characterized homogeneous protein preparations suitable for this purpose. For IL-1 $\beta$ , we have previously described methods for obtaining chemically and physically homogeneous preparations (Wingfield *et al.*, 1986, 1987b,c). In the case of IL-1 $\alpha$ , however, the recombinant-derived protein contains variable amounts of deamidated protein. Although these forms can be separated

(Wingfield *et al.*, 1987a), the possibility of further deamidation during storage and handling exists. Furthermore, even though deamidation has been shown in this report to have no major effect on receptor binding affinity and biological activity, it is clearly undesirable to have preparations with charge heterogeneity for crystallization purposes and for studying physical properties in general.

We have shown that Asn-36 of IL-1 $\alpha$  undergoes partial deamidation during its preparation from *E. coli*. This deamidation is not *E. coli* related since authentic IL-1 $\alpha$  isolated from human monocytes also contains partially deamidated Asn-36 (Cameron *et al.*, 1986). The deamidation of Asn-36 is the cause of the charge heterogeneity observed upon isoelectric focusing and titration curve analysis of the recombinant-derived and also, apparently, of the authentic IL-1 $\alpha$  (Cameron *et al.*, 1986). Substituting Asn-36 for Ser by site-specific mutagenesis resulted in the isolation of an IL-1 $\alpha$  preparation homogeneous upon isoelectric focusing, and with an isoelectric point (5.45) similar to that of unmodified wild-type IL-1 $\alpha$ . The mutant IL-1 $\alpha$  does not undergo deamidation under conditions which promote further deamidation of the wild-type protein. Thus, out of the 18 amide residues present in IL-1 $\alpha$ , 10 of which are Asn residues (Figure 6), Asn-36 appears most susceptible to deamidation.

The analysis of the mutant protein by techniques which are especially sensitive to protein tertiary structure, namely <sup>1</sup>H-n.m.r. and near-u.v. circular dichroism spectroscopy, indicated either no or minor conformational differences compared with wild-type IL-1 $\alpha$ . The unchanged receptor binding affinity and biological activity of the mutant protein also support this conclusion. The structure of the mutant IL-1 $\alpha$  is currently being studied by high-resolution two-dimensional <sup>1</sup>H-n.m.r. analysis and an attempt to prepare crystals suitable for X-ray analysis is also underway (A.M. Gronenborn and G.M. Clore, manuscript in preparation).

We have recently shown that IL-1 $\beta$  also appears to undergo a specific base-catalysed deamidation which changes the isoelectric point of the protein from 6.7 to ~5.6 (P. Wingfield, unpublished observations). We have not determined which amide residue was deamidated but have shown that it had no effect on *in vitro* biological activity. Related to this, Kronheim *et al.* (1986) described a recombinant-derived IL-1 $\beta$  with an isoelectric point similar to the aforementioned assumed once-deamidated protein. This result could have been due to the protein deamidation during the acid extraction step used in their purification procedure. The actual mechanism(s) of the IL-1-specific deamidations and any possible *in vivo* implications remain to be established.

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### References

- Ahern, T.J., Casel, J.I., Petsko, G.A. and Klibanov, A.M. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 675–679.
- Cameron, P.M., Lamyuco, G.A., Chin, J., Silberstein, L. and Schmidt, J.A. (1986) *J. Exp. Med.*, **164**, 237–250.
- Gronenborn, A.M., Clore, G.M., Schmeissner, U. and Wingfield, P. (1986) *Eur. J. Biochem.*, **161**, 37–43.
- Hunkapillar, M.W., Lujan, E., Ostrand, F. and Hood, L.E. (1983) *Methods Enzymol.*, **91**, 227–236.
- Lowenthal, J.W. and MacDonald, H.R. (1986) *J. Exp. Med.*, **164**, 1060–1074.
- MacDonald, H.R., Wingfield, P., Schmeissner, U., Shaw, A., Clore, G.M. and Gronenborn, A.M. (1986) *FEBS Lett.*, **290**, 295–298.
- March, C.J., Mosley, B., Larsen, A., Cerretti, D.B., Braedt, G., Price, V., Gillis, S., Henney, C.S., Kronheim, S.R., Grabstein, K., Conlon, P.J., Hopp, T.P. and Cosman, D. (1985) *Nature*, **315**, 641–647.
- Matthew, J.B., Friend, S.H., Botelho, L.H., Lehman, L.D., Hanania, G.I. and Gurd, F.R.N. (1978) *Biochem. Biophys. Res. Commun.*, **81**, 416–421.
- Norris, K., Norris, F., Christiansen, L. and Fill, N. (1983) *Nucleic Acids Res.*, **11**, 5103–5112.
- Kronheim, S.R., Cantrell, M.A., Deeley, M.C., March, C.J., Glackin, P.J., Anderson, D.M., Hemenway, T., Merriam, J.E., Cosman, D. and Hopp, T.P. (1986) *Bio-Technology*, **4**, 1078–1082.
- Oppenheim, J.J., Kovacs, E.J., Matsushima, K. and Durum, S.K. (1986) *Immunol. Today*, **7**, 45–56.
- Robinson, A.B. and Rudd, C.J. (1974) *Curr. Top. Cell Regul.*, **8**, 247–295.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
- Wingfield, P., Payton, M., Tavernier, J., Barnes, M., Shaw, A., Rose, K., Simona, M.G., Demaczuk, S., Williamson, K. and Dayer, J.-M. (1986) *Eur. J. Biochem.*, **160**, 491–497.
- Wingfield, P., Payton, M., Graber, P., Rose, K., Dayer, J.-M., Shaw, A. and Schmeissner, U. (1987a) *Eur. J. Biochem.*, **165**, 537–541.
- Wingfield, P., Graber, P., Rose, K., Simona, M.G. and Hughes, G.J. (1987b) *J. Chromatogr.*, **387**, 291–300.
- Wingfield, P., Graber, P., Morva, N.R., Clore, G.M., Gronenborn, A.M. and MacDonald, H.R. (1987c) *FEBS Lett.*, **215**, 160–164.

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