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CHROMATOFOCUSING OF N-TERMINALLY PROCESSED FORMS OF PROTEINS

ISOLATION AND CHARACTERIZATION OF TWO FORMS OF INTERLEUKIN-1β AND OF BOVINE GROWTH HORMONE

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SUMMARY

Using chromatofocusing, two fractions have been obtained from recombinant-derived interleukin-1β (IL-1β) and from pituitary-derived bovine growth hormone (BGH). The forms of both proteins responsible for these fractions have been characterized by N-terminal and C-terminal amino acid sequence determination. Recombinant IL-1β, as a mixture of correctly processed polypeptide and an N-terminally methionylated form, was resolved rapidly by chromatofocusing. BGH was resolved into the full-length polypeptide commencing Ala-Phe-Pro-Ala-Met-Ser-Leu- and a form truncated at the N-terminus by four amino acid residues, which thus commences Met-Ser-Leu-; the fraction containing the truncated form also contains a species having N-terminal Phe-Pro-Ala-. These results, and the possible generality of the separation, are discussed.

INTRODUCTION

The removal of N-terminal methionine from biosynthetic proteins is not always quantitative (*e.g.*, ref. 1) so a simple, rapid and general method of effecting a separation would be useful. We now demonstrate the chromatofocusing separation of recombinant-derived interleukin-1β (IL-1β) from a form from which the initiating methionine had not been removed. We attribute the success of this separation to a small difference in pK_a between N-terminal methionine and N-terminal alanine, a difference which occurs in a region of the pH scale where few functional groups on proteins have their pK_a. The small difference is thus sufficient to affect the p*I* of the protein and so permit a separation by chromatofocusing. We expect chromatofocusing to offer a useful and possibly rather general separation of recombinant-derived

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polypeptide chains from versions of the same chains which possess an initiating methionyl residue.

We have also applied the technique of chromatofocusing to the case of pituitary-derived bovine growth hormone (BGH), in an attempt to obtain rapidly and conveniently a pure, well characterized product. Even after gentle handling and an extensive purification scheme, BGH isolated from pituitaries shows two major bands on isoelectric focusing (IEF)[2]. The charge difference between these bands is less than that associated with deamidation. It has been shown[3,4] that N-terminal Ala is associated with the band of higher isoelectric point (p*I*) and that N-terminal Met (or N-terminal Phe, depending on the pH used during the extraction from pituitaries) is associated with the band of lower p*I*. Wallis[5] has shown that a difference in pK_a, sufficient to explain these separations, exists between the α-amino groups of the N-terminal phenylalanyl and alanyl chymotryptic peptides of BGH. Starting with a commercially available source of BGH, we have been able to separate two forms by chromatofocusing and to characterize them.

MATERIALS AND METHODS

Chromatofocusing of IL-1β

Recombinant-derived IL-1β (Biogen S.A.) dissolved in 25 m*M* imidazole–acetate pH 7.6 (column buffer) was applied either to a fast protein liquid chromatography (FPLC) Mono P HR5/20 column (Pharmacia; 2 mg were applied at a protein concentration of 2 mg/ml) or to a column (11 cm × 1.5 cm) containing Polybuffer exchanger 94 (21 mg protein were applied at a concentration of 7 mg/ml). Both columns were previously equilibrated with column buffer. The Mono P column was eluted at 1 ml/min at room temperature with 57 ml of a Polybuffer 96/74 mixture (20:1) diluted 1:15 in water and adjusted to pH 6.0 with acetic acid (Polybuffers from Pharmacia). The Polybuffer exchanger column was eluted at 50 ml/h at 4°C with 200 ml of Polybuffer 96 diluted 1:13 in water and adjusted to pH 6.0 with acetic acid. In order to remove Polybuffer from pooled fractions, solid ammonium sulphate was added to 82% of saturation. The precipitated protein was collected by centrifugation and dissolved in 20 m*M* ammonium bicarbonate. The clear solution was dialysed at 4°C against several changes of this buffer and then freeze-dried.

Chromatofocusing of bovine growth hormone

BGH (Batch AFP 671) isolated from bovine pituitaries was obtained as a lyophilized powder from Dr. A. F. Parlow (Harbor General Hospital, University of California Medical Centre, Torrance, CA, U.S.A.). The hormone (20 mg) was dissolved in 25 m*M* ethanolamine hydrochloride, pH 9.4 (column buffer), at a protein concentration of 4 mg/ml and was applied to a column (11 cm × 1.5 cm) containing Polybuffer exchanger 94 (Pharmacia) equilibrated with column buffer. The column was eluted with 200 ml Pharmalyte pH range 8–10.5 (Pharmacia) diluted 1:45 in water and adjusted to pH 8.0 with hydrochloric acid. The flow-rate was 50 ml/h, 1.7-ml fractions were collected and the temperature was 4°C. Column fractions (20-μl aliquots) were monitored by analytical electrofocusing as described below. Pharmalyte was removed from pooled fractions by the addition of solid ammonium sulphate to 77% saturation. The precipitated protein was collected by centrifugation,

resuspended with 1% acetic acid containing 5 *M* urea and the clear solution dialysed against several changes of 1% acetic acid at 4°C.

Analytical separation methods

Electrofocusing was carried out on thin-layer polyacrylamide gels (LKB Ampholine PAG plates, pH range 3.5–9.5) according to the manufacturer's instructions. Isoelectric points were estimated using an IEF calibration kit, range 3–10 (Pharmacia). Combined electrofocusing–electrophoresis (titration curve analysis) was performed according to the method described in ref. 6 (supplied with the LKB titration curve kit). The pH gradient was measured using a surface electrode (Bio-Rad). An Ultrophor (LKB) electrofocusing unit was used for both applications. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was carried out on 12.5% gels with a Protean I vertical gel electrophoresis system (Bio-Rad) using the buffer system described by Laemmli[7].

N-terminal amino acid sequence determination by gas chromatography–mass spectrometry (GC–MS)

This was performed according to methods previously described[8,9].

Edman degradation

Edman degradation was performed with a gas-phase sequencer (Applied Biosystems), a liquid-phase sequencer (modified Beckmann 890C) or manually. In all cases, the identification of phenylthiohydantoin (PTH) derivatives was by reversed-phase high-performance liquid chromatography (HPLC) with an internal standard for quantitation.

C-terminal amino acid sequence determination

For IL-1β, the GC–MS C-terminal sequencing method was used[9]. For BGH, the carboxypeptidase method as described by Ambler[10] was used.

Theoretical titration curve analysis

The net protein charge at a given pH value was predicted using a simple calculation procedure based on the Henderson–Hasselbach equation and which ignored electrostatic and hydrogen-bonding effects[11]. The pK_a values of titratable groups were based on those given by Matthew *et al.*[12]: C-terminal–COOH (3.6); Asp (4.0); Glu (4.5); His IL-1β (7.5); His BGH (6.3); N-terminal ^+H_3N–Met (7.5); N-terminal ^+H_3N–Ala (8.0); Cys (9.0); Tyr (10.0); Lys (10.4) and Arg (12.4). The pK_a values of the single His in IL-1β and N-terminal ^+H_3N–Met were estimated empirically, being those giving the best fit between the theoretical and experimental titration curves.

RESULTS AND DISCUSSION

Chromatofocusing of IL-1β

Prior to chromatofocusing, IL-1β consists of two components as determined by analytical IEF (Fig. 1 insert, lane a). These components were separated by chromatofocusing and called pool A and pool B (Fig. 2).

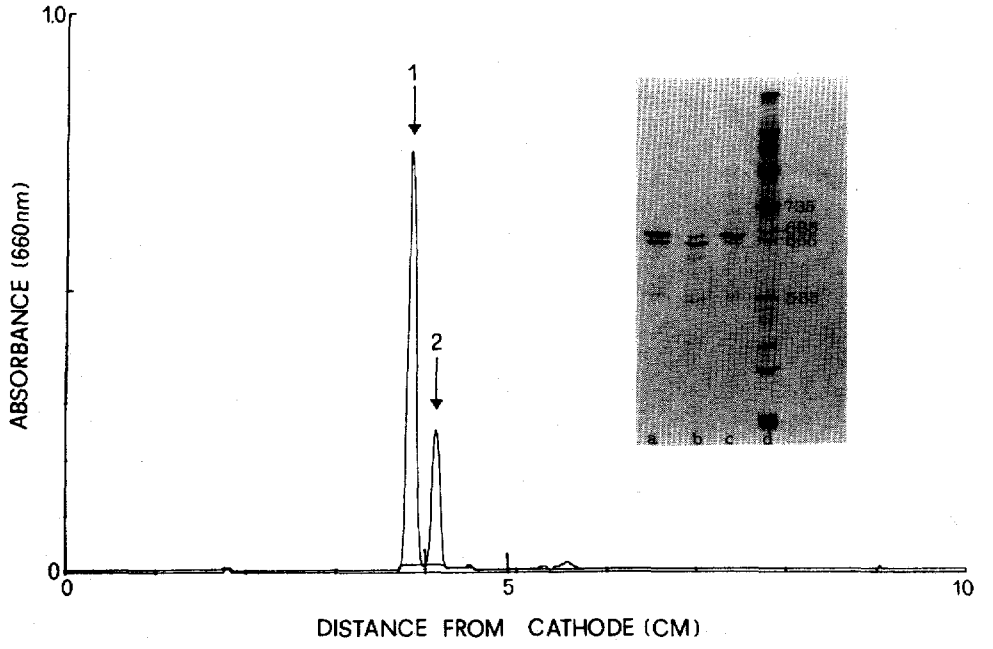

Fig. 1. Isoelectric focusing on polyacrylamide gel of samples of IL-1β: a densitometric scan of IL-1β separated by IEF as described under Materials and methods. The numbered arrows show the position of the separated polypeptides in order of decreasing isoelectric point (p*I*). The p*I* values and relative proportions of the two components, peaks 1 and 2, are p*I* 6.70 (75%) and p*I* 6.4 (25%), respectively. The insert is a photograph of an IEF gel. Lanes: c and b = pools A and B from a chromatofocusing separation on Polybuffer exchanger 94; a = unfractionated IL-1β and d = a mixture of standard proteins of known p*I* values, which are indicated.

Amino acid sequence determination of IL-1β

Gas-phase Edman degradation of IL-1β prior to chromatofocusing showed it to consist of a mixture of approximately 70% correctly processed polypeptide (commencing Ala) and approximately 30% polypeptide chain from which the initiating methionyl residue had not been removed. This ratio corresponds quite closely with that of the intensities of the bands separated by IEF (Fig. 1). C-terminal sequencing by GC–MS (data not shown) gave only one C-terminal sequence, -Phe-Val-Ser-Ser, which is expected from the DNA sequence[13]. The two forms of IL-1β were not separable by SDS-PAGE, since the difference in molecular weight is too small.

Edman degradation of the two forms of IL-1β separated by chromatofocusing showed pool A to possess N-terminal Ala-Pro and pool B to possess N-terminal Met-Ala. Since both forms derive from the same DNA construction, the separation by chromatofocusing is most likely to be due to N-terminal processing (presence or absence of N-terminal methionine) and not to any difference in the content of charged residues.

Chromatofocusing of BGH

Prior to the chromatofocusing step, BGH shows two bands on IEF (Fig. 3),

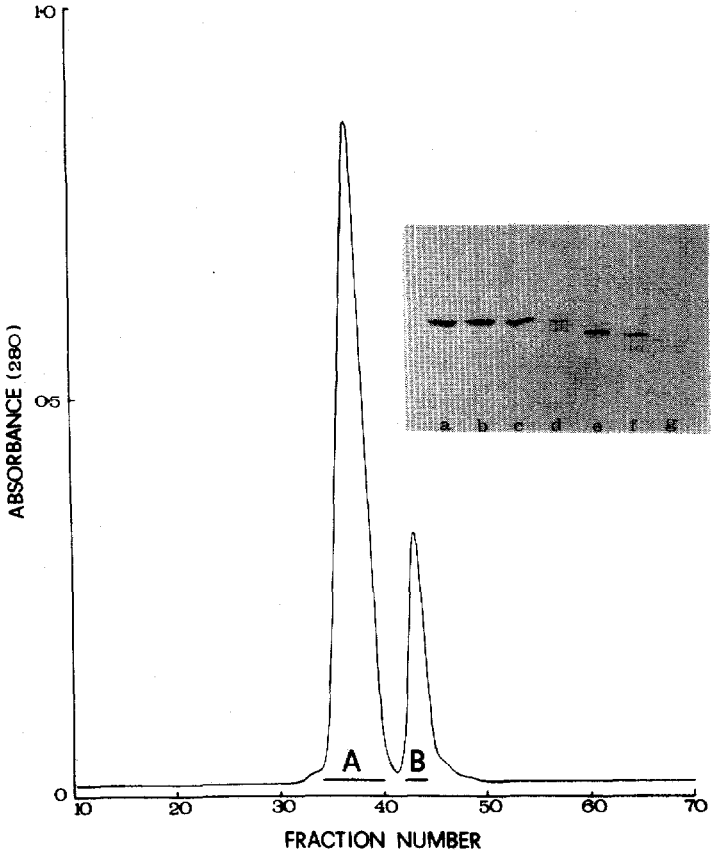

Fig. 2. Separation of two forms of IL-1β by chromatofocusing on a Mono P column as described under Materials and methods. The bars labelled A and B indicate pooled fractions. The insert shows the IEF on a polyacrylamide gel of the column fractions. Lanes a–g correspond to fractions 37–43, respectively.

as found previously (*e.g.*, ref. 2). The major band has the lower p*I*. A small difference in the apparent molecular weights of the two forms is found on SDS-PAGE (data not shown), with the major band having the lower value, in agreement with the results of Brems *et al.*[14]. When the bands are eluted from an IEF gel and subjected separately to SDS-PAGE, it is found, as expected from the relative intensities of the bands, that the band having the lower p*I* has the lower apparent molecular weight (data not shown). The two forms of the protein giving rise to these bands may be separated by chromatofocusing (Fig. 4). The separation is not complete under the conditions used, but, by appropriate pooling, material of adequate purity may be obtained. Analytical IEF (Fig. 3, lane a) and SDS-PAGE show that the first peak eluted (Fig. 4, peak A) is the one having the higher p*I* and the higher apparent molecular weight. The second peak eluted (Fig. 4, peak B) is the one having the lower p*I* (Fig. 3, lane b) and the lower apparent molecular weight. The peak from the chromatofocusing experiment which was eluted with salt (Fig. 4, lane p) consists mainly of deamidated forms of BGH (see below).

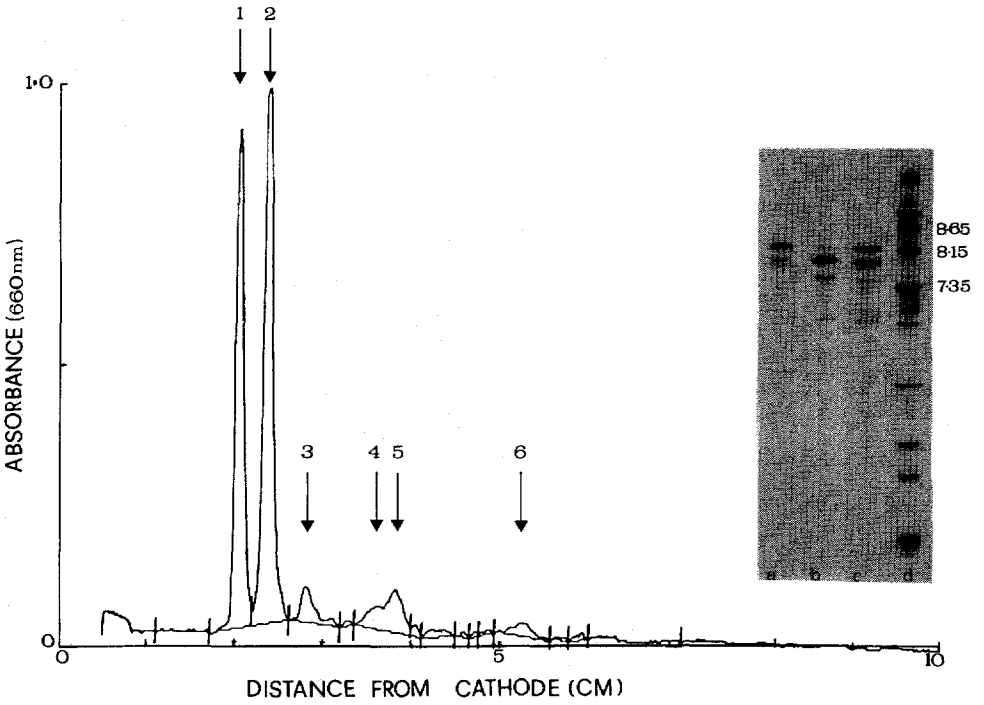

Fig. 3. Isoelectric focusing on polyacrylamide gel of samples of BGH: a densitometric scan of BGH separated by IEF as described under Materials and methods. The numbered arrows show the position of the separated polypeptides in order of decreasing isoelectric point. The isoelectric points and relative proportions of the main components, peaks 1 and 2, are p*I* 8.2 (33%) and p*I* 8.0 (45%), respectively (42 and 58% if components 3–6 are ignored). The insert is a photograph of an IEF gel. Lanes: a and b = pools A and B, respectively, from the chromatofocusing separation shown in Fig. 4, the main protein bands correspond to peaks 1 and 2 of the densitometric scan; c = unfractionated BGH and was the starting material for the chromatofocusing separation; d = a standard protein mixture of known isoelectric points, which are indicated.

Amino acid sequence determination of BGH

Edman degradation of BGH prior to the chromatofocusing step gave three N-termini: Ala (51%), Met (37%) and Phe (12%). After chromatofocusing, peak A was found by GC–MS (data not shown) to have the N-terminal sequence Ala-Phe-Pro-Ala- and peak B the N-terminal sequence Met-Ser-Ilx- (Ilx = Ile or Leu, not differentiated; Leu is expected from the DNA sequence[15]). The small cross-contamination of the two samples visible on the inserts to Figs. 3 and 4 was confirmed during the GC–MS analysis. Edman degradation of the peak A and B materials confirmed the mass spectrometric result: one cycle on peak A showed N-terminal Ala with a trace amount of N-terminal Met, and three cycles on peak B showed, in addition to the major sequence Met-Ser-Leu-, a minor sequence Phe-Pro-Ala- and a trace of Ala-Phe-Pro-. The salt-eluted chromatofocusing peak was found to contain N-terminal Ala, Met and Phe in approximately the same proportions as prior to chromatofocusing, as expected were it to contain largely deamidated forms of BGH.

The C-terminal sequence of both fractions was deduced to be -Ala-Phe, in agreement with previous work on the mixture[16].

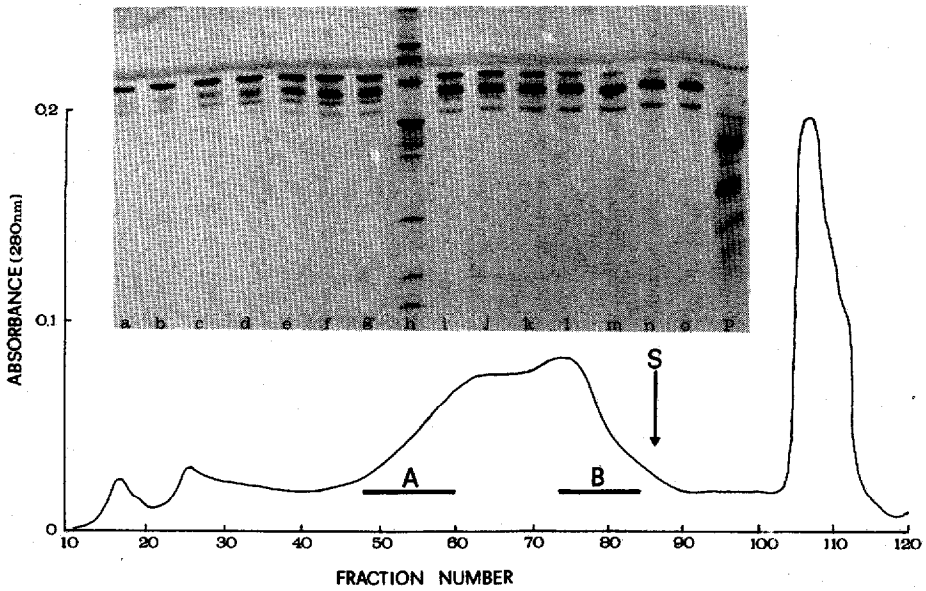

Fig. 4. Separation of two forms of BGH by chromatofocusing as described under Materials and methods. The bars labelled A and B indicate pooled fractions. An analytical IEF gel of these pools is shown in Fig. 3. The arrow S indicates the point of application of column buffer containing 1.0 *M* sodium chloride. The absorbance of the salt-eluted peak is largely due to the displacement of Pharmalyte (UV-absorbing) by the salt. The insert shows the IEF on a polyacrylamide gel of the column fractions. Lanes: a–o and p = even-numbered column fractions 52–78 and 107, respectively; h = a standard mixture of proteins of known p*I* values corresponding to the values shown in Fig. 1 (the standard of p*I* 3.50 is not shown here).

Peak A thus corresponds with the full-length polypeptide as predicted from the DNA sequence[15] and assuming the presence of a signal peptide with cleavage of the polypeptide chain between Gly and Ala. The major N-terminal sequence of peak B, Met-Ser-Leu-, appears once in the predicted sequence and corresponds to the polypeptide truncated by a further four residues. The slightly lower predicted molecular weight for the truncated form (peak B) is in accord with the SDS-PAGE result (data not shown).

Previous studies on BGH which had not been purified as described here have demonstrated N-terminal heterogeneity (see, *e.g.*, refs. 3–5). Our results extend this work by obtaining more than just an N-terminal residue (important when peptide impurities are thought to be present—see Ellis *et al.*[3]) and obtaining a C-terminal sequence.

pK_a of N-terminal residues

We attribute the separations shown in Figs. 1–4 to differences in pK_a of the various N-terminal α-amino groups. The methionyl polypeptides have the lower p*I* values. Such an explanation of the p*I* difference is in accord with previous work on BGH (cited above). Differences in amino terminal pK_a are common: recent work involving NMR spectroscopy of semisynthetic myoglobins[17] shows that a large difference in amino terminal pK_a exists between (Gly[1])myoglobin (pK_a 7.72) and (Leu[1])myoglobin (pK_a 7.15). For these differences to permit a separation based on

protein p*I*, it is helpful[5] if the protein p*I* is close to the amino terminal pK_a values, a region relatively distant from the pK_a values of most of the ionizable groups in proteins. IL-1β is a particularly favourable case, since it has only one His residue.

The effect of the pK_a of the N-terminal residue on the p*I* of the protein is well illustrated in Fig. 5 for IL-1β, and a similar result was obtained for BGH (data not shown).

CONCLUSIONS

Since chromatofocusing separates proteins on the basis of differences in p*I*, such separations of N-terminally processed polypeptides should be a general phenomenon wherever a sufficient difference in p*I* is created. In such cases, chromatofocusing would offer a means of separating target biosynthetic polypeptides from forms from which the initiating methionyl residue has not been removed, as described in this paper for the case of recombinant-derived IL-1β. Chromatofocusing could be used on an analytical scale to screen for conditions minimizing the production of methionylated material, and to provide pure samples for small scale trials. Scaling-up operations may be possible, and in this regard it is interesting to note the use of cheaper buffers for chromatofocusing[18].

In addition, using the methods described in this paper it is possible to prepare, on a milligram scale and from commercially available starting material, full-length BGH of defined primary structure. Such material should be useful for physicochemical studies and studies of biological activity in various systems. A considerable amount of work has recently been performed with heterogeneous material (*e.g.*, refs. 2, 14). In contrast to the preparative IEF separations of Ellis *et al.*[3], which take

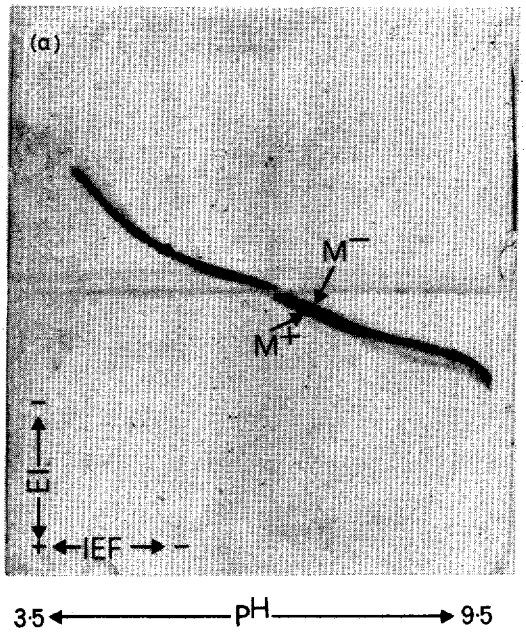

Fig. 5.

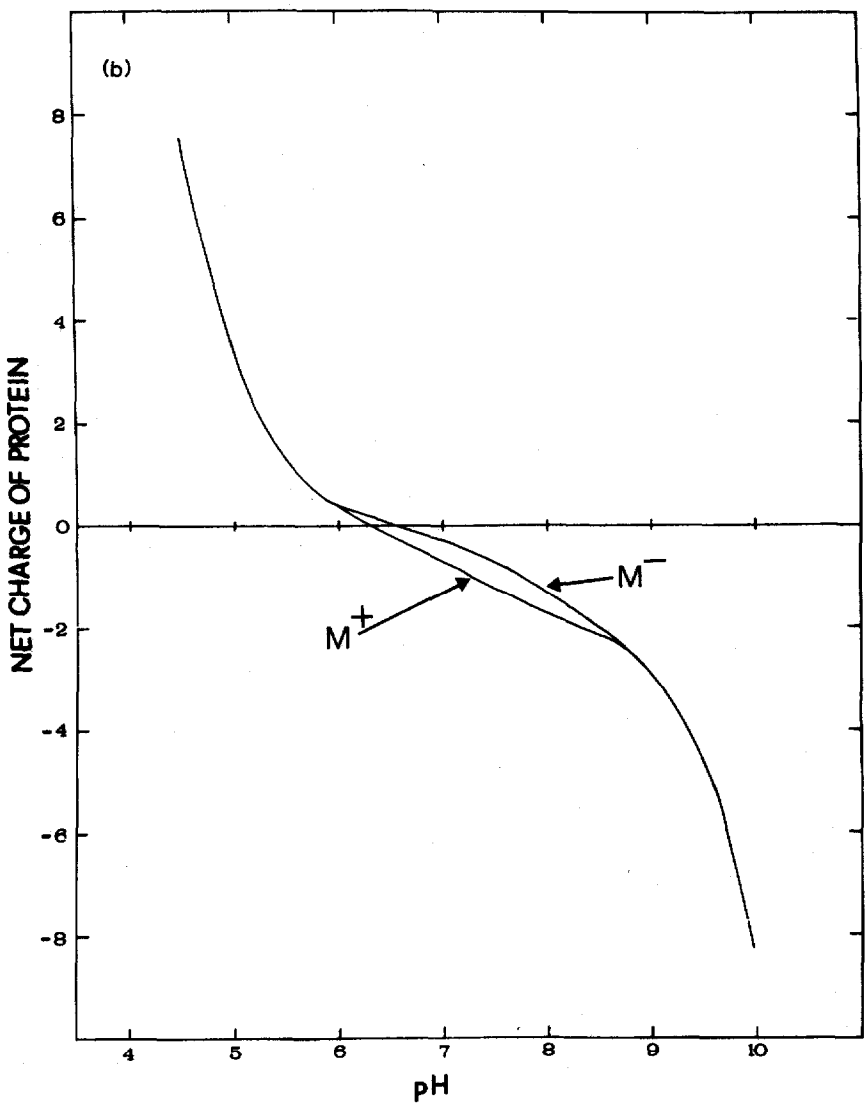

Fig. 5. (a) Titration curve of IL-1β over the range pH 3.5–9.5. Conditions for the titration curve analysis are described under Materials and methods. In the lower left corner, the two double arrows with positive and negative symbols indicate the direction and polarity of isoelectric focusing (IEF) and electrophoresis (EI). The arrows labelled M^- (p*I* 6.70) and M^+ (p*I* 6.4) indicate the separated forms of IL-1β in which the N-terminal residue is either alanine or methionine, respectively. (b) Theoretical titration curve of IL-1β, generated as described under Materials and methods. The labelled arrows M^- and M^+ indicate predicted titration curves using a pK_a of either 8.0 or 7.5 for the N-terminal H_3N^+ group. A pK_a of 7.5 for the single His residue was used for both curves. The predicted p*I* values of the M^+ and M^- species are 6.25 and 6.55, respectively. This compares with the corresponding experimental values of 6.40 and 6.70 (Figs. 1 and 5a).

2–4 days, the chromatofocusing purification is easily performed in a relatively short period of time, about 60 min using the FPLC Mono P column or 3–4 h using the low-pressure Polybuffer exchanger column.

Whilst this paper was in preparation, Yamada *et al.*[19] reported the separation by chromatofocusing of recombinant-derived interleukin-2 from its methionylated form. Here we extend these findings to two further proteins and provide an explanation for the separations achieved. The feasibility of such separations can readily be tested for other proteins by using the experimental and theoretical titration curve analysis described herein.

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