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Specific Nitrogen-15 Labelling of Leucine Residues in Human Growth Hormone

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Biosynthetic human growth hormone (hGH) specifically ^{15}N labelled in the leucine residues has been obtained by recombinant DNA technology, using ^{15}N-labelled leucine and an *E. coli* strain that requires leucine. It is shown that, despite the possibility of minor transaminase activity, the labelling on the whole is specific, and that the two-dimensional ^{1}H–^{15}N correlation NMR spectra of hGH can be greatly simplified by this methodology.

In NMR[†] studies of the structure and the structure–function relationship of larger proteins, the availability of specifically isotope-labelled proteins is essential in order to obtain NMR spectra that are sufficiently simple, and to allow sequence specific assignment techniques to be applied.[1–3] For this purpose proteins are being used that are labelled either with ^{15}N in all nitrogen positions or with ^{13}C in all carbon positions, or uniformly labelled in both types of position.

Further simplifications can be obtained if the isotope labelling can be confined to specific types of amino acid residues. Thus, recently it has been demonstrated[4] that biosynthetic human growth hormone (hGH), specifically ^{13}C-labelled in the carbonyl position of all 26 leucine residues, can be obtained in large quantities by means of recombinant DNA techniques, and that NMR studies[5] of this species can provide detailed information on the tertiary interactions in the hormone.

Here we investigate the possibility of synthesizing hGH specifically ^{15}N-labelled in the amide groups of the 26 leucine residues, using recombinant DNA techniques. This ^{15}N-labelled species is of particular interest in the sequential assignment of the ^{1}H NMR spectrum of the hormone. However, because of possible transaminations the synthesis in itself constitutes a challenge.[2]

Experimental

Fermentation of E. coli *in the presence of* $(^{15}NH_4)_2SO_4$. Authentic-sequence hGH was made by means of recombinant DNA techniques essentially as outlined previously.[6] *Escherichia coli* (*E. coli*), strain MC1061, was employed[7] which, *inter alia*, requires the amino acid leucine (MC1061/pHD86-3SP13). Fermentation was carried out in a defined salt medium with $(^{15}NH_4)_2SO_4$ as the dominant nitrogen source (3.5 g l^{-1}, ICON, 99,5% ^{15}N), and with leucine as the only amino acid added to the medium. An aqueous solution of acetic acid (475 g l^{-1}) and glucose (25 g l^{-1}) was used as the carbon source. The fermentation (4 l) was carried out as a carbon-limited fed batch process at 30°C and a pH of 7.2. Leucine was added gradually during the fermentation in order to minimize the metabolism of leucine. Thus, a total of 0.1 g l^{-1} leucine was added before inoculation as well as after 24 h, while 0.2 g l^{-1} was added 48 h after inoculation. The yield of hGH was 150 mg l^{-1} after 72 h. Following the fermentation, the cells were harvested and mechanically homogenized at 350 bar followed by cell-debris removal and sterile filtration. As the leucines account for only 6% of the total amount of added nitrogen, the lower limit of the ^{15}N labelling is 94% on a average.

In order to determine the possible incorporation of ^{15}N in the leucine residues, the *E. coli* broth was hydrolyzed in 6 M HCl. After removal of the hydrochloric acid the

[†] Abbreviations used in the text: NMR, nuclear magnetic resonance; hGH, human growth hormone; ^{13}C, carbon-13; DNA, deoxyribonucleic acid; ^{1}H, hydrogen-1 or proton; *E. coli*, *Escherichia coli*; ^{15}N, nitrogen-15; SDS, sodium dodecyl sulphate; IEF, isoelectric focusing; FPLC, fast protein liquid chromatography; GP-HPLC, gel permeation high-performance liquid chromatography; SQC, single quantum coherence; NH, backbone amide proton; TSP, 2,2,3,3-tetra-deuterio-3-(trimethylsilyl)propionic acid.

amino acids were derivatized with phenyl isothiocyanate according to the Pico–Tag method.[8] The leucine derivative was collected, and converted into the corresponding phenylthiohydantoin derivative followed by molecular weight determination by mass spectrometry. The mass spectra showed an ^{15}N incorporation in the leucine residues of at least 50% (*vide infra*).

Purification of uniformly ^{15}N-labelled hGH. The amino acid extension, used in the applied method, was specifically removed by means of the enzyme cathepsin C. The purification of the protein and the removal of the *N*-terminal extension was done as outlined previously.[6,7] The product was of high purity as judged from SDS-Page and IEF electrophoresis. After purification the protein was lyophilized in a glycine–sodium bicarbonate buffer. The lyophilized material, approximately 22 mg, was dissolved in 7.5 ml of distilled water and desalted against water, employing three PD-10 columns (Pharmacia). The desalted samples were pooled, lyophilized and dissolved in a small amount of distilled water containing 10% D_2O (Norsk Hydro, 99.8% deuterium). After adjustment of the pH to 2.66 with 1 M hydrochloric acid, the solution became clear. Subsequently the protein solution was transferred to a 5 mm NMR tube. The estimated concentration of hGH was 25 mg ml^{-1}. No charge variants were present as judged by IEF. This was confirmed by FPLC analysis employing a Mono-Q column (Pharmacia). SDS-Page showed no mass variants. A GP–HPLC analysis showed a 1.2% content of dimer.

Fermentation of E. coli *in the presence of ^{15}N-leucine.* The fermentation was carried out with the *E. coli* strain, mentioned above, in a defined salt medium supplemented with the L-form of the amino acids alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine, all at a concentration of 0.5 g l^{-1}. A solution of glucose (500 g l^{-1}) was used as the carbon source. The fermentation (3 l) was carried out as described for the uniformly labelled hGH. A total of 0.35 g l^{-1} ^{15}N-leucine, (Cambridge Isotope Laboratories, 99% ^{15}N) was added before inoculation together with the other amino acids, and 0.35 g l^{-1} was added after 18 h of fermentation. The yield of hGH was 100 mg l^{-1} after 43 h. The cells were harvested and homogenized as described for the uniformly labelled hGH. Approximately 50% of the leucine residues were ^{15}N-labelled according to an amino acid analysis and molecular weight determination by mass spectrometry.

Purification of hGH ^{15}N-labelled in the leucine residues. Since minor amounts of impurities do not influence the NMR spectra significantly, the purification procedure outlined in Refs. 6 and 7 was simplified by omission of the hydrophobic interaction chromatography and the gel-filtration, normally applied in order to remove dimers and other aggregates. The preparation of the NMR samples were carried out as described for the uniformly labelled sample. The pH was adjusted to 2.63 and small amounts of precipitate were removed by centrifugation and decantation. Analysis by GP–HPLC showed only traces of dimers and polymers. No deamidated product could be detected by IEF and silver staining.

NMR measurements. The NMR experiments were run at a temperature of 32 °C on a Bruker AM500 spectrometer operating at a ^{1}H resonance frequency of 500.13 MHz and a ^{15}N resonance frequency of 50.68 MHz. A total of

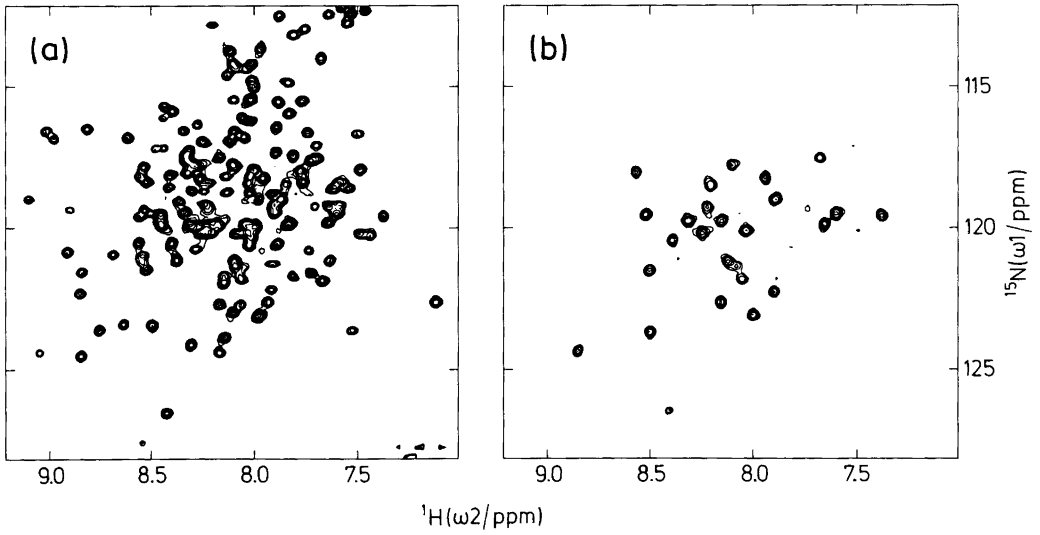

Fig. 1. A region of the ^{1}H–^{15}N correlation NMR spectra of (a) uniformly labelled hGH and of (b) hGH specifically ^{15}N labelled in the leucine residues. The ^{1}H chemical shift is expressed relative to TSP and measured with respect to the water signal at 4.69 ppm. The ^{15}N chemical shift is expressed relative to the signal of liquid ammonia and measured with respect to the water signal analogous to the procedure employed in Ref. 14.

600 µl of protein solution, approximately 25 mg ml^{-1}, was contained in the 5 mm NMR sample tube. Proton-detected two-dimensional ^{1}H–^{15}N correlation spectra (Fig. 1) were recorded using an SQC-type experimental scheme[9] with composite pulse decoupling of ^{15}N during the acquisition period.[10,11] In the time domain, the recorded data sets consisted of 256 free induction decays each represented by 2048 real data points. The acquisition times in the t_1 and t_2 dimensions were 99.4 ms and 155.6 ms, respectively. The total recording time was 12 h for the spectrum recorded on the uniformly labelled sample. A total of 128 transients were averaged for each t_1 value. For the sample specifically labelled in the leucines the recording time was 40 h, while 320 transients were averaged for each t_1 value. The two spectra were processed identically before the hypercomplex Fourier transformation. Thus, a Lorenzian–Gaussian filtering function was applied in the t_2 dimension, while a shifted squared sinebell filtering function and zero filling to 1024 points were applied in the t_1 dimension.

Results and discussion

In *E. coli* K12 at least 12 transport systems for the uptake of individual amino acids or groups of structurally related amino acids have been kinetically defined.[12] This allows the simultaneous transport of the supplied amino acids into the amino acid pool of the *E. coli* cell in sufficient amounts until the depletion of the most frequently used amino acids. In general, the presence of the amino acids represses amino acid synthesis by a feedback mechanism. Also a high amount of ammonia in the medium, as well as the use of glucose as the carbon source throughout the fermentation (*vide infra*), represses amino acid catabolism.[13] As a consequence the transaminase activity is expected to be low.

Fig. 1(a) and 1(b) show the two-dimensional ^{1}H–^{15}N correlation spectra of uniformly ^{15}N-labelled hGH, and of hGH specifically labelled with ^{15}N in the leucine residues, respectively. The spectrum in Fig. 1(b) accounts for all the 26 leucine residues in hGH, as the spectrum contains 26 well-resolved and intense signals. The large NH and ^{15}N chemical shift dispersions for the leucine amide groups indicate strong correlations between the chemical shift values and the local environments of the leucine residues, implying that the secondary and tertiary structures of the protein is preserved. This is in accordance with our previous studies[4] of ^{13}C-labelled hGH.

The simplification of the NMR spectra obtained by specific ^{15}N-labelling of the leucines is striking when comparing Fig. 1(a) and 1(b). The massive overlap in the spectrum of uniformly labelled hGH in Fig. 1(a) is efficiently removed in the spectrum of the specifically ^{15}N-labelled hGH in Fig. 1(b), because of the absence of correlations from residues other than leucine. Although cross-labelling of ^{15}N to other types of residue does take place by metabolic processes, the level is rather low. Thus, at lower contour levels (not shown) a number of weak signals corresponding to other types of residue appear. A comparison of these signal intensities with those shown in Fig. 1(b) indicates that the incorporation of ^{15}N into any other residue types is less than 20% of that of leucine.

On the other hand, the low signal-to-noise ratio of the spectrum of the specifically labelled sample compared with the signal-to-noise ratio of the uniformly labelled sample, together with the longer recording time, suggests a general dilution of the ^{15}N on leucine. This is in accordance with the result from mass spectrometry, mentioned above, that only about 50% of the leucines retained their ^{15}N-labelling.

In the case of uniformly ^{15}N-labelled hGH, fermented with $(^{15}NH_4)_2SO_4$ as the sole ^{15}N source, and with ^{14}N-leucine added to the medium in order to comply with the auxotrophicity for leucine of the bacterial strain employed, the ^{15}N cross-labelling to the leucine residues was also considerable despite the gradual addition of leucine (*vide supra*). A comparison of the intensities of the NMR signals from the leucine residues with those from other residue types shows no significant differences. That is, the level of ^{15}N isotope labelling of the leucine residues relative to that of other residue types is at least 80%. This should be compared with a mass spectrometric measurement which indicates a ^{15}N-incorporation of at least 50%. The main reason for the dilution of the isotope is most likely transaminase activity that causes scrambling of the α-amino groups between different types of amino acid.

In summary the procedure presented here shows that biosynthetic human growth hormone, specifically ^{15}N-labelled in all 26 leucine residues, can be obtained even though some scrambling occurs during the fermentation. However, the scrambling is still sufficiently low to allow an unambiguous distinction of the ^{1}H–^{15}N correlations of the leucine residues from those of the other types of residue in the protein.

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