Specific Carbon-13 Labelling of Leucine Residues in Human Growth Hormone

Thorkild Christensen,[a] Ejnar B. Jensen,[a] Flemming Junker,[a] Henrik Dalbøge,[a] Frits Abildgaard[b] and Jens J. Led[b]

[a] Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd, Denmark and [b] Department of Chemistry, University of Copenhagen, The H. C. Ørsted Institute, Universitetsparken 5, DK-2100 Copenhagen Ø, Denmark

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Biosynthetic human growth hormone specifically ^{13}C-labelled in the carbonyl positions of all 26 leucine residues has been obtained by recombinant DNA techniques using ^{13}C-labelled leucine and an *E. coli* strain that requires leucine. It is shown that, on the whole, the labelling is specific with no significant mislabelling as would have been the case had the ^{13}C-labelled leucine been metabolized.

In the last few years highly purified proteins have become available in relatively large quantities through recombinant DNA techniques. So far these techniques have been valuable particularly in the pharmaceutical industry where they are being used commercially in the production of important biologically active proteins such as human insulin and human growth hormone (hGH). In addition, the new technologies have made it possible to prepare analogues of proteins[1] that are important for the study of their function.

Another area of increasing importance for protein synthesis by recombinant DNA is related to the NMR spectroscopic determination of the solution structure of proteins. Today, NMR spectroscopy provides a method complementary to single-crystal diffraction analysis for modelling of the three-dimensional structure of proteins[2] by allowing a determination of the solution structure of the proteins. Moreover, unlike the X-ray crystallographic method, the NMR approach does not require the availability of well-defined crystals. Owing to the complexity of the NMR spectra of proteins, detailed solution structures of only relatively small (MW $\leq$ 15 kDa) and non-aggregating proteins can be determined by NMR spectroscopy at present. In order to increase this limit it is therefore a challenge to the NMR spectroscopist further to develop the NMR method. This development includes appropriate isotope labelling of the protein. Thus isotope-labelled derivatives allow the assignment of the complex proton NMR spectra of proteins by providing spectra that are simpler than those obtainable from unlabelled proteins. Also, the labelled derivatives result in a reduction of the time necessary to record the spectra by increasing the sensitivity.

Here we describe the preparation of biosynthetic human growth hormone (BhGH) specifically labelled with ^{13}C in carbonyl carbon of the leucine residues, and demonstrate that specific labelling, free of mislabelled derivatives, can be obtained by recombinant DNA technology.

Materials and methods

Fermentation of E. coli *in the presence of ^{14}C-leucine.* *Escherichia coli (E. coli),* strain MC1061 was used[3] which, *inter alia,* requires the amino acid leucine (MC1061/pHD86-3SP13). Small-scale cultures for pilot labelling with ^{14}C-labelled leucine were grown overnight in 5 ml LB ampicillin media. Two cultures were grown in parallel. An aliquot of the cultures was diluted in 200 ml minimal medium containing 50 mM ampicillin, and was grown to an absorbance A = 0.05. One millilitre of ^{14}C-Leu (50 μCi, NEN) was added and incubated at 37 °C for 16 h. Samples were collected from the cultures by centrifugation. After mechanical lysis, at total amino acid analysis was carried out on the lysed product without further purification using the Pico Tag System.[4] During the amino acid analysis the individual phenyl isothiocyanate amino acids were collected and transferred to scintillation vials with scintillator (Aquazol 2). The ^{14}C-content was analysed on a β-scintillation counter.

Fermentation of E. coli *in the presence of ^{13}C-leucine.* Authentic-sequence human growth hormone (hGH) was made by means of recombinant DNA techniques, essentially as outlined previously.[5] Fermentation with ^{13}C-labelled leucine was carried out with the above mentioned *E. coli* strain in a defined salt medium, with [1-^{13}C]leucine, (99 % Stohler/Kor) as the only amino acid added to the medium. Fermentation (10 l) was carried out as a limited-glucose fed batch process at 30 °C, and pH 7.2. ^{13}C-Leucine was continously added at a rate of 0.01 g l^{-1} h^{-1} during the whole fermentation, using a total of 0.5 g l^{-1}. The hGH

yield was 100 mg l^{-1}. After the fermentation the cells were harvested and mechanically homogenized at 600 bar followed by cell-debris removal and sterile filtration.

The purification and removal of the amino-terminal extension is described elsewhere.[3,5] To the purification process was added an isoelectric precipitation as the last step.

Preparation of sample for NMR analysis. A Sephadex G25 M column (2×25 cm) was equilibrated with a buffer containing 20 mg ml^{-1} perdeuteriated glycine (ICN Biochemical 98 %) and 10 mM $Na_2DPO_4 \cdot 2D_2O$. The pH was adjusted to 7.5 with NaOD. As a liquid only D_2O more than 99 % deuteriated (Norsk Hydro) was used. The precipitate of the purified product was dissolved in 7 ml of buffer. A total of 143 mg was applied to the column and eluted with buffer at a flow rate of 1.6 ml min^{-1}. A total of 135 mg ^{13}C-labelled biosynthetic hGH was recovered in a concentration of 12.5 mg ml^{-1}. As judged by IE-HPLC, using a Pharmacia Mono Q column HR 5/5, the product was more than 99 % pure.[3] Five millilitres of the ^{13}C-labelled BhGH solution were transferred to an NMR tube.

Results and discussion

The ^{14}C-Leu labelling experiment was carried out to investigate whether leucine was metabolized during the fermentation procedure. In Table 1 are shown the results of a total amino acid analysis on whole cells from *E. coli* strain MC1061/pHD 86-3SP13 grown on a medium containing ^{14}C-Leu. The peaks for the individual amino acids were collected and examined for content of ^{14}C by scintillation counting. As shown by the data, the majority of radioactivity is present in the leucine peak, indicating that, on the whole, the added ^{14}C-Leu remains unmetabolized. The increased level of radioactivity in the isoleucine peak as compared with the other amino acids is most probably due to a partial overlap of the leucine and isoleucine peaks (approximately 2 % overlap; chromatogram not shown). The radioactivity found in the peak for lysine cannot be attributed to the separation method but is rather due to metabolism of leucine. Thus, leucine may be metabolized to acetyl-CoA which is directly involved in the synthesis of lysine. Furthermore, Acetyl-CoA enters into the citric acid cycle, which may explain the slightly elevated level of ^{14}C in all the amino acids, as compared with ammonia. Except for lysine, the amount of radioactivity incorporated into amino acids other than leucine is minimal. The same level of radioactive incorporation was observed in both experiments.

Table 1. ^{14}C-Scintillation counting,[a] culture MC1061/pHD 86-3SP13.[b]

Amino acid	Culture 1	Culture 2
Ala	39	34
Asp + Glu	95	111
Phe	43	50
Gly + His	25	71
Ile	1045	1109
Lys	266	265
Leu	25200	42206
Met	84	31
Pro	32	32
Arg	61	37
Ser	27	35
Thr	40	47
Val	44	57
Tyr	46	39
NH_3 (Std)	29	22

[a] ^{14}C Cpm per 100 µl PITC-amino acid. [b] PITC-Amino acid collected during amino acid analysis.

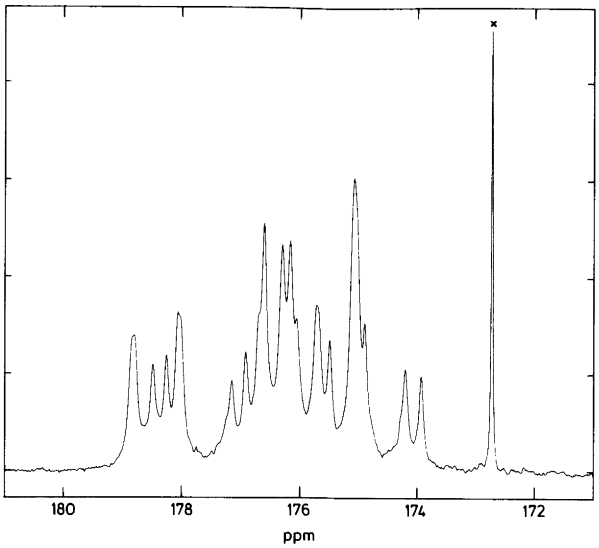

Fig. 1. The carbonyl region of the NMR spectrum of human growth hormone ^{13}C-labelled in the carbonyl groups of the 26 Leu residues of the hormone. The spectrum was obtained from a 0.8 mM solution of hGH in D_2O at pD 7.5, containing 20 mg ml^{-1} perdeuteriated glycine buffer. The chemical shift is expressed relative to external Me_4Si and was measured with respect to the ^{13}C signal of the carbonyl carbon of glycine (×) at 172.7 ppm.

Fig. 1 shows the carbonyl region of the ^{13}C spectrum of ^{13}C-labelled BhGH. In other regions of the ^{13}C spectrum only signals corresponding to ^{13}C in natural abundance were observed. As shown recently[6] the NMR spectrum can account for all 26 leucine residues present in BhGH. The relatively large chemical shift range covered by the carbonyl resonances is striking, considering the fact that the resonances represent only Leu carbonyl carbons. This dispersion indicates a significant dependence of the observed chemical shift on the secondary and tertiary structure of the hormone, and suggests that detailed information about these structures, including the dynamics of the locations of the individual Leu residues, can be obtained from NMR studies of the ^{13}C-labelled BhGH. Such studies are in progress and will be published elsewhere.

In summary, the procedure presented here shows that specifically ^{13}C-labelled BhGH of high purity can be

obtained in large quantities by means of recombinant DNA techniques, and it is demonstrated that a specific ^{13}C-labelling can be introduced into a biosynthetic protein. By appropriate selection of starting material, other labels can be specifically introduced by a similar procedure. Thus labelling with radioactive isotopes or other stable isotopes of interest in NMR studies, such as ^{15}N or ^{2}H, can be effected, including mixed specific isotope labelling. The *E. coli* strain (MC1061) used, which requires leucine to be supplied, also enables the uniform isotope labelling of BhGH, except in the leucine residues. Moreover, by selecting different *E. coli* strains that require other amino acids, different types of labelling can be obtained. Finally, labelled proteins of the type described here may also be valuable in the elucidation of metabolic pathways.

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