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A novel ^{15}N-labeling method to selectively observe $^{15}NH_2$ resonances of proteins in ^{1}H-detected heteronuclear correlation spectroscopy

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An experimental method to selectively label side-chain NH_2 groups of glutamine and asparagine in proteins with ^{15}N is proposed. This selective labeling method enables to observe only $^{15}NH_2$ resonances and thus, to discriminate between ^{15}NH and $^{15}NH_2$ resonances in a ^{1}H-detected heteronuclear correlation spectrum. This method gives results with approximately two times higher sensitivity than those obtained by elaborate pulse sequences such as DEPT-HSQC and will be useful for studying the molecular interaction involving the side chains of Asn and Gln residues.

^{1}H-detected heteronuclear correlation spectroscopy; ^{15}N-labeling; Acidic fibroblast growth factor

1. INTRODUCTION

For the analysis of NMR spectra of proteins with molecular weights greater than 15 kDa, ^{1}H-detected ^{15}N heteronuclear correlation spectroscopy aided by stable isotope labeling methods is now an essential technique [1–3]. In such a spectrum, some of amide ^{15}NH and $^{15}NH_2$ resonances overlap with each other so the experimental methods which separate these types of resonances are required. Pulse sequences such as DEPT-HMQC or DEPT-HSQC have been applied for this purpose [4–7]. However, due to longer delays, the experiments with these pulse sequences are less sensitive than those with HMQC or HSQC. As an alternative to these pulse techniques, we report a novel method for selective labeling of $^{15}NH_2$ groups in proteins, where we take acidic fibroblast growth factor (aFGF, M = 15 kDa) as an example.

2. MATERIALS AND METHODS

2.1. *Sample preparation*

For the preparation of ^{15}N-uniformly-labeled aFGF, *E. coli*, the strain JM103, having the expression plasmid for human aFGF, pMJ26, was cultured in M9 minimal medium containing 99% ^{15}N-enriched NH_4Cl (Isotec inc.). For preparing $^{15}NH_2$-selectively labeled aFGF, the ^{15}N-enriched M9 medium was supplemented with non-labeled amino acids in the following recipe: 400 mg alanine, 400 mg arginine, 250 mg aspartic acid, 50 mg cystine, 400 mg glutamic acid, 400 mg glycine, 100 mg histidine, 100 mg isoleucine, 100 mg leucine, 100 mg lysine, 250 mg methionine, 50 mg phenylalanine, 100 mg proline, 1.0 g serine, 100 mg threonine, 50 mg tryptophan, 100 mg tyrosine, and 100 mg valine [8]. It should be noted here that in this recipe glutamine and asparagine are not included, although 400 mg glutamine is used in the original recipe described in [8]. ^{15}N-uniformly or $^{15}NH_2$-selectively-labeled aFGFs were purified according to the protocol described previously [9].

Abbreviations: HMQC, heteronuclear multiple quantum coherence; HSQC, heteronuclear single quantum coherence; DEPT, distortionless enhancement of polarization transfer; aFGF, acidic fibroblast growth factor.

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2.2. *NMR experiments*

The sample for NMR contained 1.5 mM aFGF in 10 mM sodium d_3-acetate buffer, pH* 5.6, with 500 mM sodium chloride in 90% H_2O/10% D_2O (pH* values were pH meter direct reading). The pulse sequence used for measuring HSQC spectra was the same as described previously [10]. The delay needed for the coherence transfer was set to 2.1 ms, which is slightly shorter than $1/4J_{NH}$. For the DEPT-HSQC experiment [7], the delay corresponding to $1/2J_{NH}$ was set to 4.2 ms. 180° pulse was applied as the third proton pulse, which separates $^{15}NH_2$ from ^{15}NH resonances in opposite signs, respectively [7]. A short spin-lock pulse, 1.5 ms, was applied prior to acquisition for purging water resonance in both HSQC and DEPT-HSQC experiments [10]. All spectra were measured at 24°C on a JEOL JNM-GX400 spectrometer operating at 400 MHz for protons. The size of each FID matrix was as follows: for the HSQC and DEPT-HSQC spectra of ^{15}N-uniformly-labeled aFGF, 1024 × 256 complex points ($t_2 \times t_1$) with 2181.6 Hz F_1 spectral width, and 1024 × 128 complex points with 1400 Hz F_1 spectral width were used, respectively. For the measurement of the HSQC spectrum of $^{15}NH_2$-selectively-labeled aFGF, 1024 × 256 complex points with 1400 Hz F_1 spectral width were used. For all 2D spectra, one zero-filling in t_1 dimension was applied prior to the Fourier transformation. Acquisition times per each t_1 increment were 128 for the HSQC spectrum, 256 for the DEPT-HSQC spectrum of the ^{15}N-uniformly-labeled aFGF and 128 for the HSQC spectrum of the $^{15}NH_2$-selectively-labeled aFGF.

3. RESULTS AND DISCUSSION

Fig. 1a shows the HSQC spectrum of aFGF uniformly labeled with ^{15}N. In this spectrum, some of the

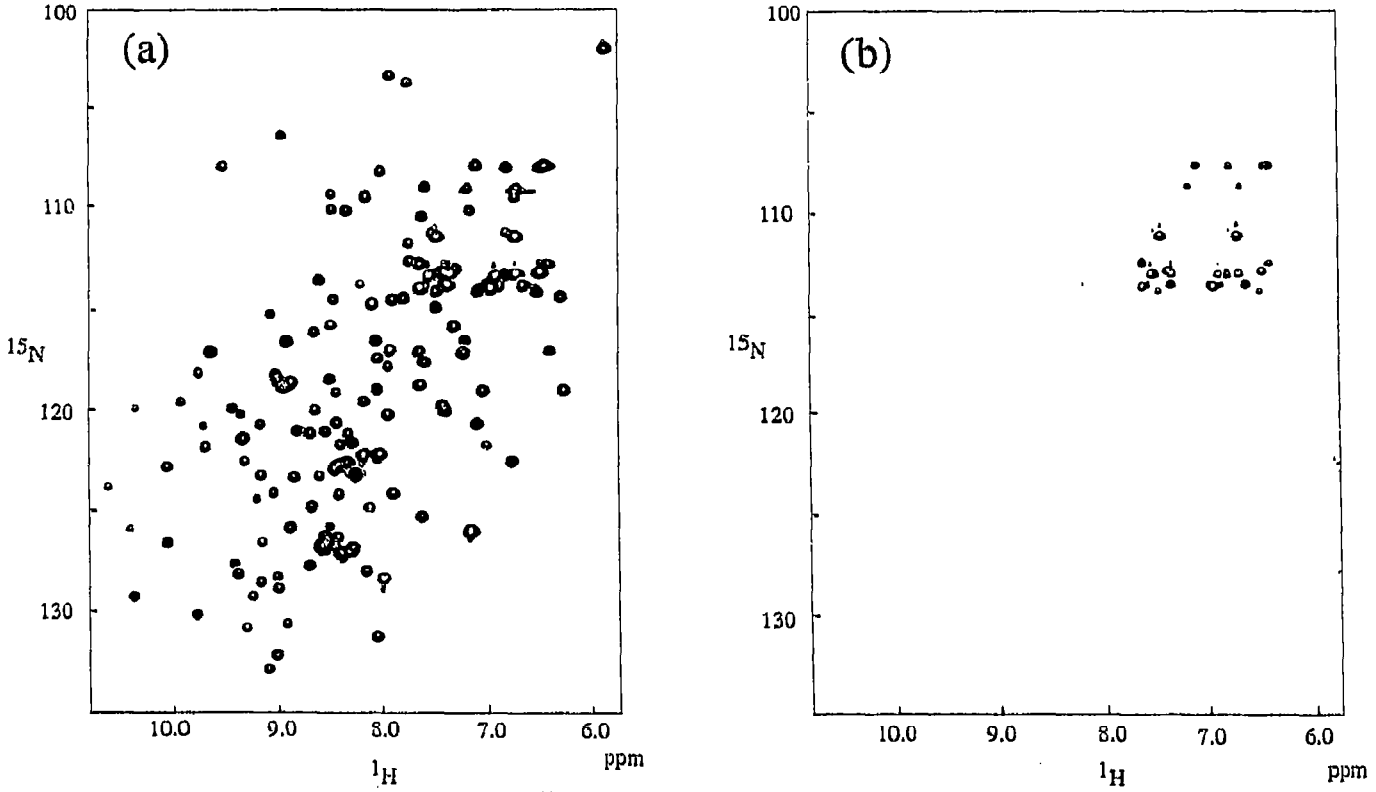

Fig. 1. The HSQC spectra of (a) ^{15}N-uniformly-labeled, and (b) $^{15}NH_2$-selectively-labeled aFGF.

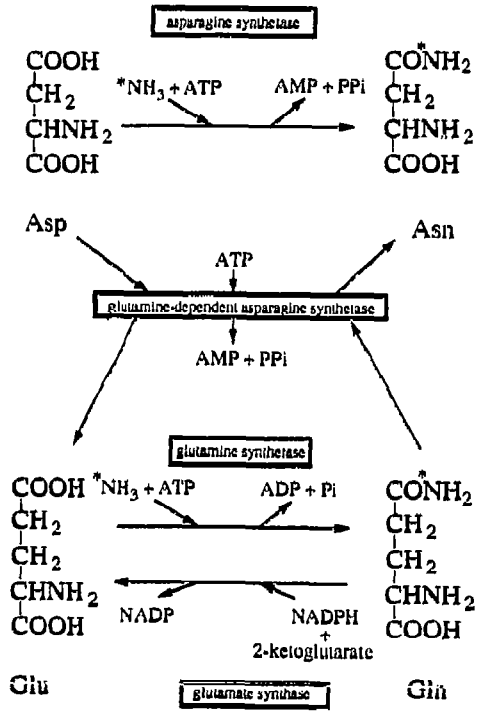

Fig. 2. The metabolic pathways among asparagine, aspartic acid, glutamine and glutamic acid. The ^{15}N nuclei incorporated from a culture medium are marked with asterisks.

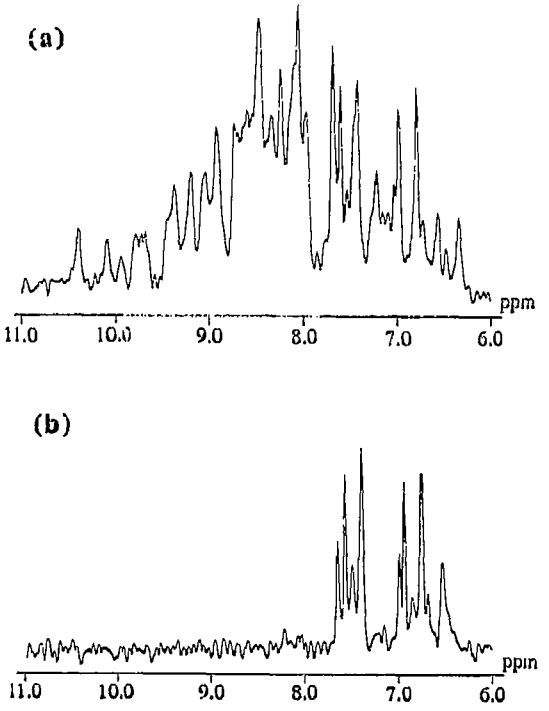

Fig. 3. The first increment data of HSQC of (a) ^{15}N-uniformly-labeled aFGF and (b) $^{15}NH_2$-selectively-labeled aFGF. Both data were measured with $t_1 = 400\,\mu s$, 1K complex data points for FID, spectral width = 6000 Hz, acquisition times = 128. The data were processed by the use of $\pi/4$ shifted sine bell.

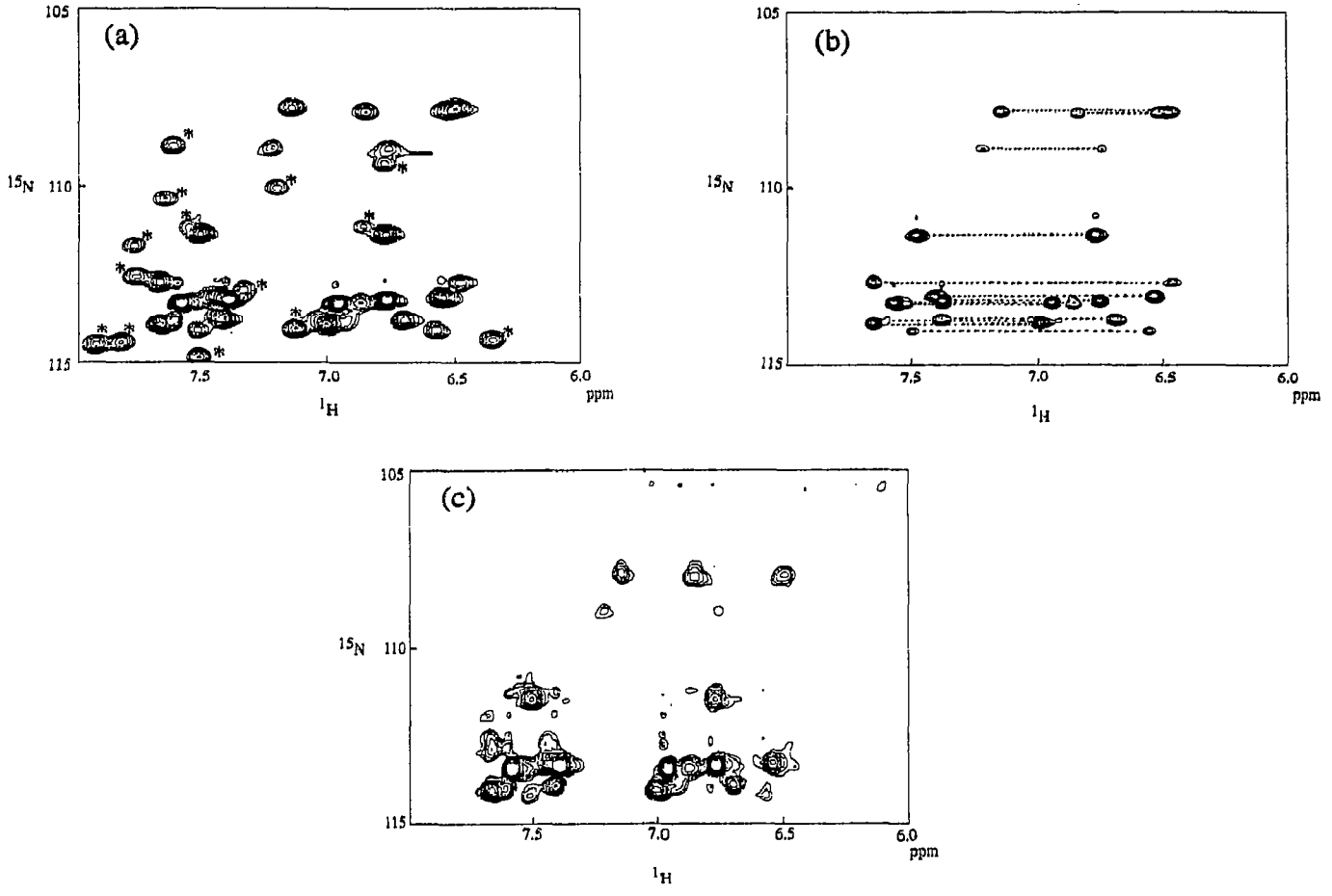

Fig. 4. The expanded region of HSQC spectra of (a) ^{15}N-uniformly-labeled aFGF, (b) $^{15}NH_2$-selectively-labeled aFGF, and (c) the corresponding region of the DETP-HSQC spectrum of ^{15}N-uniformly-labeled aFGF. ^{15}NH resonances are shown with asterisks in (a) and the 13 pairs of the $^{15}NH_2$ resonances are connected by dotted lines in (b). In the DEPT-HSQC experiment, the third proton pulse was set to 180°, and only the plane containing $^{15}NH_2$ resonances is displayed.

amide ^{15}NH and $^{15}NH_2$ correlation peaks overlap with each other so that discrimination between these peaks is essential for the assignment purpose. DEPT-HSQC [7] is applicable for this purpose. However, as an alternative way, we propose a novel method to label NH_2 groups selectively with ^{15}N in the present communication. The biosynthetic pathways of Asn and Gln are shown in Fig. 2 [11]. COOH groups in Glu are converted to $CONH_2$ of Gln by glutamine synthetase by incorporating ammonia from the growth medium in an ATP-dependent manner. In the case of Asn, its $CONH_2$ group is synthesized in two ways: one, from Asp by glutamine-independent asparagine synthetase and the other, from Gln by glutamine-dependent asparagine synthetase [11]. Therefore, under the condition that ammonium chloride labeled with ^{15}N and sufficient amounts of non-labeled amino acids are added in the growth medium, we expect selective labeling of the $CONH_2$ groups with ^{15}N in Gln and Asn residues. In Fig. 1b, we show the HSQC spectrum of aFGF purified from *E. coli* grown in the minimal medium containing $^{15}NH_4Cl$ supplemented with non-labeled amino acids. The spectrum in Fig. 1b is essentially the same as that obtained with DEPT-HSQC, demonstrating that the NH_2 groups in aFGF are selectively labeled with ^{15}N (both expanded spectra are shown in Fig. 4b and c). Fig. 3 demonstrates the selectivity of ^{15}N labeling to the NH_2 groups with this procedure. Both data were taken from the first increment of the HSQC spectra of (a) ^{15}N-uniformly-labeled and (b) $^{15}NH_2$-selectively-labeled aFGF observed under the same experimental conditions. Although the intensities of the NH_2 signals in Fig. 3b are almost the same as those in Fig. 3a, the background ^{15}NH signals are completely eliminated (Fig. 3b), showing that specific labeling of $^{15}NH_2$ groups is accomplished in the present experimental condition.

For detailed inspection of the effect of the $^{15}NH_2$-selectively-labeling method, the HSQC spectra of the $^{15}NH_2$ region for ^{15}N-uniformly-labeled and $^{15}NH_2$-selectively-labeled aFGF samples are compared in Fig.

4a and b. On careful comparison of these spectra, we can easily identify the ^{15}NH resonances as shown with asterisks in Fig. 4a and the pairs of $^{15}NH_2$ resonances which are connected by dotted lines in Fig. 4b. In Fig. 4b, 13 pairs of signals are observed which are expected from the amino acid composition of aFGF, seven Asn and six Gln residues.

Here, we compare the sensitivity of the DEPT-HSQC spectrum of ^{15}N-uniformly-labeled aFGF (Fig. 4c) with that of the HSQC spectrum of the $^{15}NH_2$-selectively-labeled sample (Fig. 4b). The DEPT-HSQC pulse sequence requires twice as long as the total delay time compared with that of HSQC. In the case of a large molecular-weight protein, this longer delay time leads to appreciable decay of the signals before acquisition due to the short T_2 relaxation time of the magnetization. Moreover, the increase in the number of pulses used in DEPT-HSQC causes the sensitivity reduction due to accumulation of the pulse imperfection. In fact, intensity of the typical NH_2 signal measured in the DEPT-HSQC spectrum is almost half of that of the corresponding signal in the HSQC spectrum (data not shown).

As an extension of the present experiment, the possibility of the exclusive labeling of the NH_2 group either in Asn or Gln should be discussed here. In the HSQC spectrum of aFGF purified from *E. coli* grown in the culture medium described above supplemented with 400 mg non-labeled Gln, all 13 pairs of NH_2 signals were observed. Thus in this condition, NH_2 in Gln residues was also labeled with ^{15}N. This may be due to the high turn-over rate of glutamine in the metabolism of *E. coli* [11]. On the other hand, the two biosynthetic pathways of Asn (Fig. 2) are known to be inhibited by asparagine [11]. Therefore, the use of growth medium supplemented with sufficient amounts of Asn would make it possible to label only NH_2 in Gln. This experiment is now in progress in our laboratory.

Finally, we must discuss whether this NH_2-selective-labeling method is commonly used in any expression system using *E. coli*. In general, when the ammonium-ion concentration of the growth medium is sufficiently high (greater than 1 mM), ammonia is incorporated directly into the amino group of glutamate, the amide group of glutamine, and also the amide group of asparagine. Since glutamate and glutamine are used as the nitrogen source for other biomolecules such as DNA [11], the cyclic reaction pathways between Gln and Glu shown in Fig. 2, are responsible for this basic ammonia assimilation [11]. Thus, as long as we use a wild type of the *E. coli* strain which has not deficiency or mutation of the genes responsible for this ammonia assimilation reaction, this selective labeling method would work well, although we have not yet tried other strains than JM103.

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