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A novel way of amino acid-specific assignment in ^{1}H-^{15}N HSQC spectra with a wheat germ cell-free protein synthesis system

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Abstract

For high-throughput protein structural analyses, it is indispensable to develop a reliable protein overexpression system. Although many protein overexpression systems, such as ones utilizing *E. coli* cells, have been developed, a lot of proteins functioning in solution still were synthesized as insoluble forms. Recently, a novel wheat germ cell-free protein synthesis system was developed, and many of such proteins were synthesized as soluble forms. This means that the applicability of this protein synthesis method to determination of the functional structures of soluble proteins. In our previous work, we synthesized ^{15}N-labeled proteins with this wheat germ cell-free system, and confirmed this applicability on the basis of the strong similarity between the ^{1}H-^{15}N HSQC spectra for native proteins and the corresponding ones for synthesized ones.

In this study, we developed a convenient and reliable method for amino acid selective assignment in ^{1}H-^{15}N HSQC spectra of proteins, using several inhibitors for transaminases and glutamine synthase in the process of protein synthesis. Amino acid selective assignment in ^{1}H-^{15}N HSQC spectra is a powerful means to monitor the features of proteins, such as folding, intermolecular interactions and so on. This is also the first direct experimental evidence of the presence of active transaminases and glutamine synthase in wheat germ extracts.

Abbreviation: HSQC – heteronuclear single quantum coherence spectroscopy

Introduction

With the increase in the available sequence information on the genomes in various cells, the structures, properties, and functional activities of proteins (products of genes) have been settled in the midst of post-genomics and named proteomics. However, rapid progress in this scientific field requires the availability of sufficient amounts of proteins. Three major strategies are currently used for protein production: chemical synthesis, *in vivo* expression, and cell-free synthesis. The first two methods have serious problems that can not be overlooked. Chemical synthesis is difficult for long-peptide synthesis (Blaschke et al., 2000), and *in vivo* expression systems can produce proteins that do not have any significant effect on the physiology of the host cells (Golf and Goldberg, 1987; Chrunyk et al., 1993). On the other hand, with a cell-free translation system, one can synthesize larger proteins at the same or higher speed, and as accurately

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as ones for *in vivo* translation (Kurland, 1982; Pavlov and Ehrenberg, 1996), and express proteins that would interfere with the host cell physiology.

One of the most convenient and reliable eukaryotic cell-free translation systems is based on wheat germ embryos containing all the components for translation in a concentrated dried state and ready for protein synthesis after germination. A previous study indicated that such systems are generally unstable and thus insufficient (Roberts and Paterson, 1973). Recently, however, we found that plants contain endogeneous inhibitors of translation, and that in the case of conventional wheat germ extracts, the RNA N-glycosidase tritin and other inhibitors, such as thionin, ribonucleases, deoxyribonucleases and proteases, found in the endosperm inhibit translation (Ogasawara et al., 1999; Madin et al., 2000). Extensive washing of wheat embryos, in order to eliminate endosperm contamination, gives a highly stable and active extract, and with this, the protein synthesis reaction can last over 60 h (Madin et al., 2000). With a dialysis bag with continuous feeding of substrates and removal of small byproducts (Spirin et al., 1988), enzymatically active proteins can be obtained in mg quantities per ml reaction volume (Madin et al., 2000). In our previous study (Morita et al., 2003), we showed that this cell-free protein synthesis system is applicable to the comparison of protein structures and functions. This applicability is crucial for modern proteomics, which require a high-throughput means. However, for precise and high-throughput analysis of the interrelationships between the structures and functions of proteins, it is indispensable to assign the signals observed in ^{1}H-^{15}N HSQC spectra in a high-throughput means. In the case of protein synthesis systems involving *E. coli*, many scientists have developed methods for amino acid-selective labeling (Muchmore et al., 1989; Kigawa et al., 1995; Waugh, 1996). However, these methods are complicated. On the other hand, in the case of a wheat germ cell-free system, our newly developed method is much easier to perform. In this article, we report a novel and reliable method for the amino acid-specific assignment of the signals observed in the ^{1}H-^{15}N HSQC spectra of proteins synthesized with a wheat germ cell-free system. With this method, it will be much easier to identify the protein-ligand interaction surfaces, and to clarify the function of each amino acid residue in a protein, with the introduction of point mutations.

Furthermore, with this method, for Ala, Glu and Asp specific labeling, it is indispensable to use inhibitors of transaminases and glutamine synthase, and this indicates that several tranaminases and glutamine synthase mediating the inter-conversion between Ala and Glu, Glu and Asp, and Glu and Gln are active in wheat germ extracts.

Materials and methods

Synthesis of mRNA

Two mRNAs encoding RbpA1 and yeast ubiquitin were synthesized by the same method as that described in our previous paper (Morita et al., 2003).

Protein synthesis

Wheat germ extract was purchased from Cell-Free Science Company, and other reagents (other than labeled amino-acids) were purchased from Nacalai Tesque. Labeled amino acids in cell-free grade were purchased from Nihon Sanso. Inhibitors for transaminases (β-chloro-L-alanine and aminooxyacetate) were purchased from Sigma, and dissolved in water to prepare the stock solutions of inhibitors (100 mM and 10 mM for β-chloro-L-alanine and aminooxyacetate, respectively). An inhibitor for glutamine synthase, L-methionine sulfoximine was purchased from Nacalai Tesque, and dissolved in water to prepare the stock solution (1 mM).

RbpA1

Four hundred μg of synthesized mRNA was precipitated with ethanol and dissolved in 600 μl dialysis buffer, and then mixed with a wheat germ extract for protein synthesis (Madin et al., 2000). This mixture was dialyzed against the dialysis buffer containing 20 amino acids (19 non-labeled and one ^{15}N-labeled; 30 μM each) for 2 days. If necessary, inhibitors of transaminases were added to the dialysis buffer of appropriate concentrations. After protein synthesis, the reaction mixture (1.2 ml) was treated with benzonase nuclease (100 units) at 37 °C for 1 h, and then purified by ion-exchange column chromatography on an Q–sepharose FF column (5 ml), followed by size exclusion chromatography on a HiLoad Superdex 75pg gel-filtration column. The purified RbpA1 was centrifuged and concentrated with an Ultrafree-CL 5kD (Millipore^{TM}) to 50 μM protein concentration.

Ubiquitin

The 100 μg of synthesized mRNA was precipitated with ethanol and dissolved in 130 μl dialysis buffer, and then mixed with a wheat germ extract for protein synthesis (Madin et al., 2000). This mixture was dialyzed against the dialysis buffer containing 20 amino acids (19 non-labeled and one ^{15}N-labeled; 30 μM each) for 4 days. If necessary, inhibitors of transaminases were added to the dialysis buffer of appropriate concentrations. After protein synthesis, the reaction mixture (1.0 ml) was concentrated to 250 μl up to 100 μM protein concentration, then the buffer was changed to NMR buffer (50 mM sodium phosphate, 100 mM NaCl, pH 6.5) by passage through a Micro Spin G-25 column (Pharmacia) equilibrated with the same buffer. Ubiquitin was not further purified because it shows good ^{1}H-^{15}N HSQC spectra without purification (Morita et al., 2003).

Measurement of HSQC spectra

D_2O (final concentration, 10%) was added to the concentrated protein solutions (50 μM and 100 μM for RbpA1 and ubiquitin, respectively). ^{1}H-^{15}N HSQC spectra (64 (t1) × 512 (t2) complex points, 1024 scans or 256 scans) were obtained with a DMX-500 (Bruker) FT-NMR spectrometer. The data were processed using NMRPipe (Delaglio et al., 1995) on a Linux workstation. The ^{1}H and ^{15}N chemical shifts were referenced according to the method of Wishart et al. (1995). The assignments of the amide resonances of the ^{1}H-^{15}N HSQC spectra for RbpA1 and ubiquitin were used for data analyses according to previous papers reported, respectively (Morita et al., 2000; Sakamoto et al., 1999).

Results

To check the specificity of amino acid-selective labeling with the wheat germ cell-free protein synthesis system, we synthesized RbpA1s and ubiquitins with selectively labeled amino acid buffer in which one amino acid was ^{15}N-labeled and the others non-labeled, and then obtained ^{1}H-^{15}N HSQC spectra. In the sequence of RbpA1, Cys and His are not included, and for the selective labeling experiment with these we used the basic helix-loop-helix domain of PHO4 (Shimizu et al., 1997). We observed that amino acids other than Ala, Glu, and Asp are selectively labeled (data not shown). However, in the case of Ala, Glu, and Asp labeling, signals corresponding to Ala, Glu and Asp were all observed in each spectrum (Figure 1). To complete the selective labeling with Ala, Glu, and Asp, we tried to inhibit the transaminases functioning in the wheat germ extract.

To inhibit the Ala to Glu conversion, we synthesized the RbpA1 in the presence of 7 mM β-chloro-L-alanine, which is known as an inhibitor of alanine aminotranferase, and only signals corresponding to Ala were observed in ^{1}H-^{15}N HSQC spectra (Figure 2a). In the presence of β-chloro-L-alanine, the total amount of RbpA1 synthesized was almost the same as that of normally synthesized ones. However, even in the presence of β-chloro-L-alanine, signals corresponding to Glu and Asp were observed at the same time in both ^{1}H-^{15}N HSQC spectra for Glu and Asp selective labeling (Figure 2b and c), which shows that β-chloro-L-alanine did not inhibit the transaminases mediating the amino acid conversion between Glu and Asp.

To inhibit the amino acid conversions between Glu and Asp, we used aminooxyacetate, which is known as an inhibitor of aspartate aminotranferase.

In the case of RbpA1, signals corresponding to Asp were observed in the ^{1}H-^{15}N HSQC spectrum of RbpA1 for Asp selective labeling in the presence of 0.4 mM aminooxyacetate. However, signals corresponding to Ala and Glu were observed in the ^{1}H-^{15}N HSQC spectrum for Glu selective labeling, which indicated that aminooxyacetate did not inhibit alanine aminotransferase effectively at 0.4 mM concentration. Furthermore, the total amount of synthesized RbpA1 was about 30% lower than that of normally synthesized ones, judging from the gel electrophoresis results (data not shown). To improve this point, we synthesized RbpA1 in the presence of 0.4 mM aminooxyacetate and 2 mM β-chloro-L-alanine, simultaneously. In the presence of 0.4 mM aminooxyacetate and 2 mM β-chloro-L-alanine, the total amount of RbpA1 synthesized was almost the same as that of normally synthesized ones, and Glu and Asp were selectively labeled, respectively (Figures 3a and b).

On the other hand, in the case of ubiquitin, no loss of the amount of synthesized ubiquitin was observed even in the presence of 1.0 mM aminooxyacetate. At this concentration, the amino acid conversion between Ala and Glu, Glu and Asp were both inhibited (Figures 3c–e). In this case, however, Gln signals were observed in ^{1}H-^{15}N HSQC spectra for Glu selective

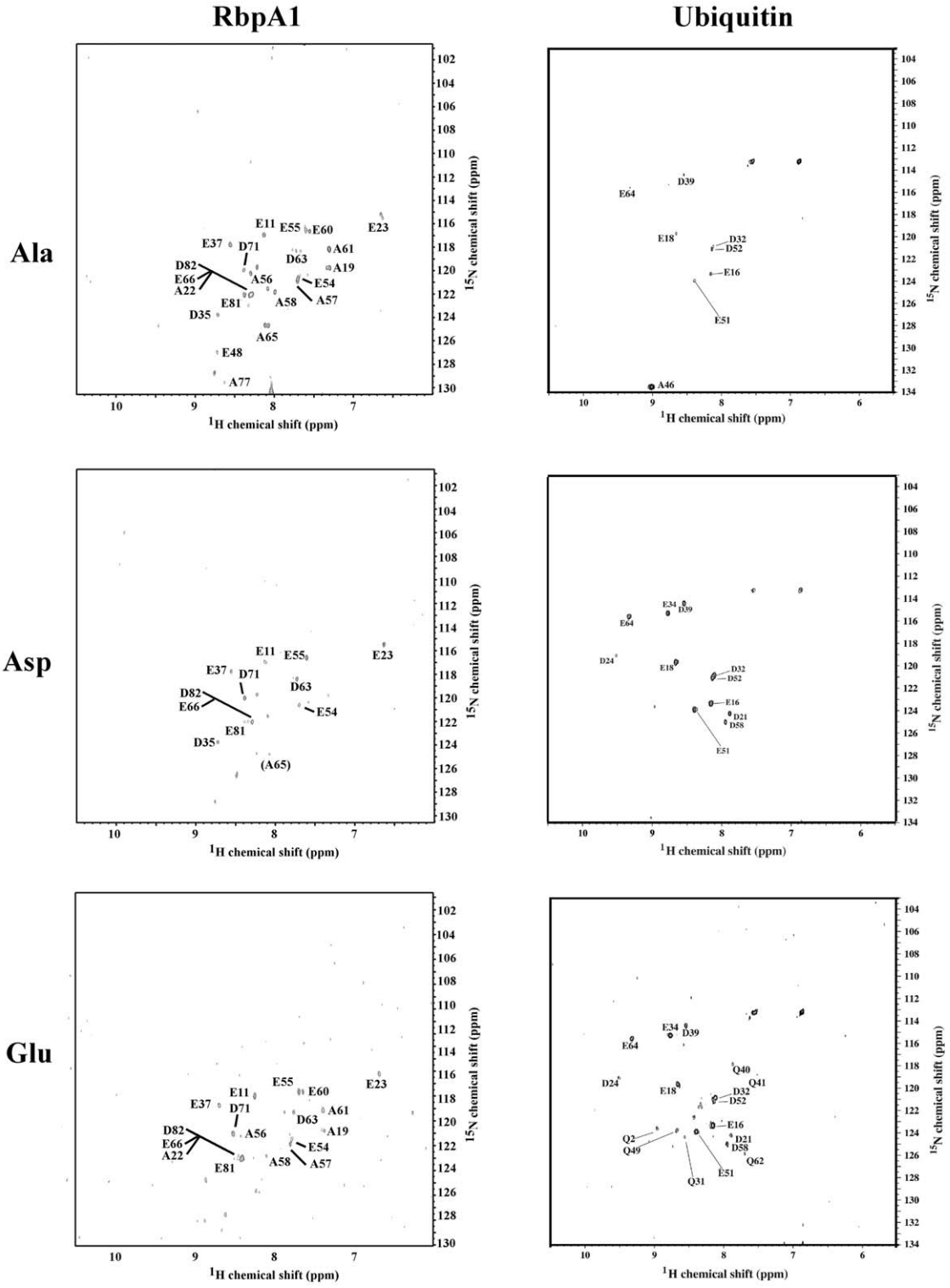

Figure 1. ^{1}H-^{15}N HSQC spectra of ^{15}N-Ala(top), ^{15}N-Asp(middle) and ^{15}N-Glu(bottom) selectively labeled RbpA1 (left columns) and yeast ubiquitin (right columns) (64 (t1) × 512 (t2) complex points, 1024 scans) obtained at the ^{1}H resonance frequency of 500 MHz. To optimize the resolution in the nitrogen dimension, ^{15}N spectral widths of 1500 Hz and 1600 Hz were used for RbpA1 and ubiquitin, respectively.

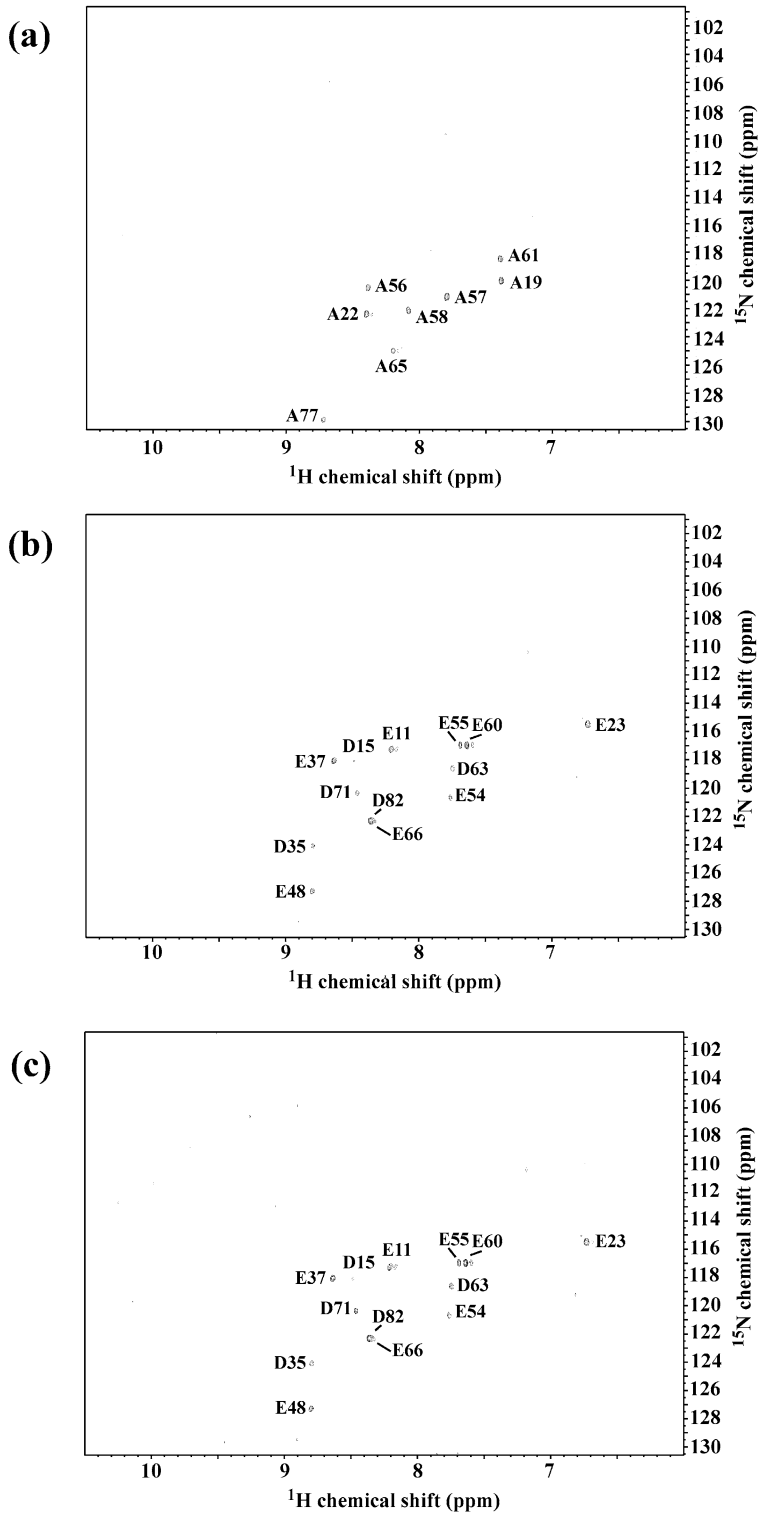

Figure 2. ^{1}H-^{15}N HSQC spectra (NMR buffer, pH 6.9, 30 °C) of RbpA1s synthesized with selectively labeled amino acid mixtures ((a)^{15}N-Ala, (b)^{15}N-Glu, and (c)^{15}N-Asp) and 7 mM β-chloro-L-alanine. The conditions for spectral measurements are the same as in Figure 1.

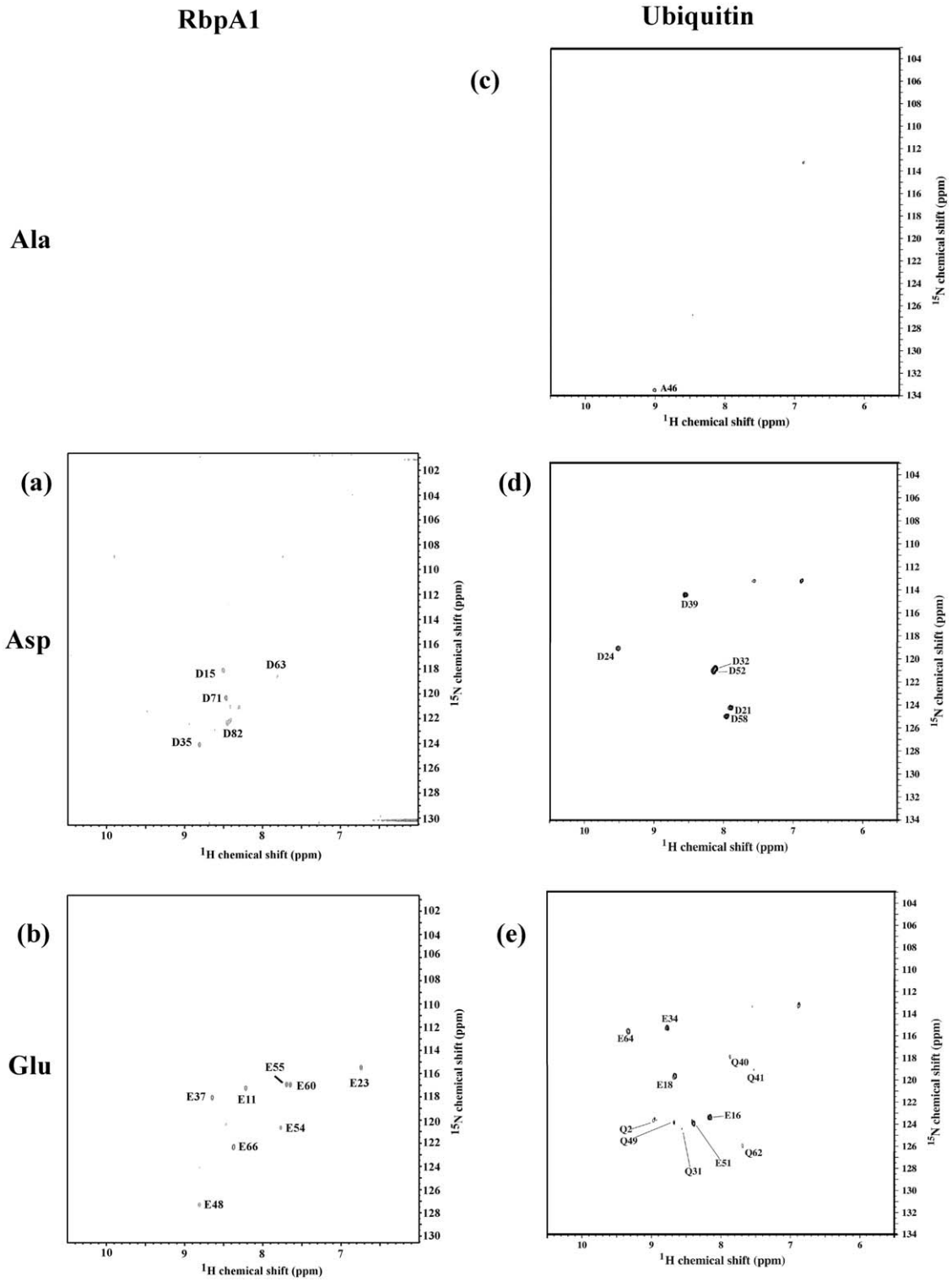

Figure 3. ^{1}H-^{15}N HSQC spectra (NMR buffer, pH 6.9, 30 °C) of RbpA1s synthesized with selectively labeled amino acid mixtures ((a)^{15}N-Glu, and (b)^{15}N-Asp), and 0.4 mM aminooxyacetate and 2 mM β-chloro-L-alanine, and ^{1}H-^{15}N HSQC spectra (NMR buffer, pH 6.5, 30 °C) of synthesized ubiquitins with selectively labeled amino acid mixtures ((c)^{15}N-Ala, (d)^{15}N-Glu, and (e)^{15}N-Asp) and 1.0 mM aminooxyacetate. The conditions for spectral measurements are the same as in Figure 1.

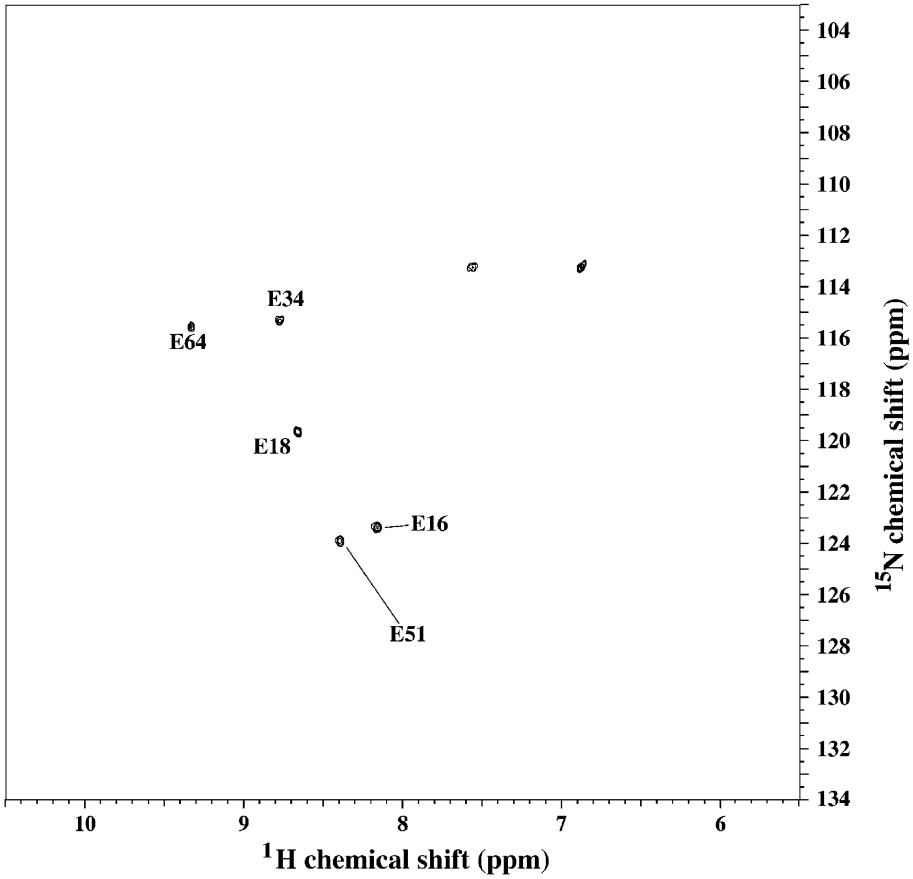

Figure 4. ^{1}H-^{15}N HSQC spectra (NMR buffer, pH 6.5, 30 °C) of synthesized ubiquitin with ^{15}N-Glu selectively labeled amino acid mixtures, 1.0 mM aminooxyacetate and 0.1 mM L-methionine sulfoximine. Conditions for spectral measurements are same as those in Figure 1.

labeling in the presence of 1.0 mM aminooxyacetate. In order to inhibit the amino acid conversion, we tried L-methionine sulfoximine, known as an inhibitor of glutamine synthase. In the presence of 1.0 mM aminooxyacetate and 0.1 mM L-methionine sulfoximine, Glu residues were selectively labeled completely (Figure 4).

On the basis of these results, we found that alanine and aspartate aminotransferases, and the glutamine synthase are active in wheat germ extracts, and developed a novel, convenient and reliable method for identifying the signals observed in ^{1}H-^{15}N HSQC spectra in a high-throughput manner (Figure 5).

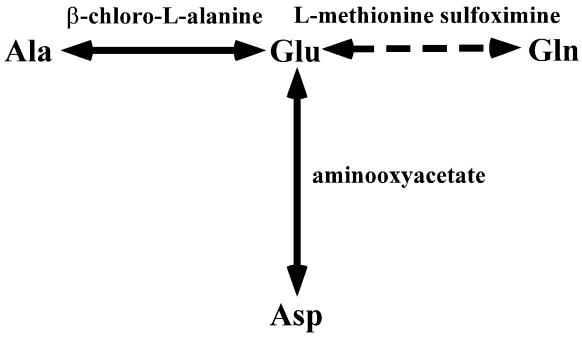

Figure 5. Summary of the inhibitors of transaminases activated in the wheat germ extract.

Discussion

We chose two proteins, RbpA1 and ubiquitin, to test the applicability of newly developed amino acid selective labeling technique. For proteins synthesized with the selectively ^{15}N-labeled amino acid mixtures other than the ^{15}N-Ala, ^{15}N-Asp, and ^{15}N-Glu ones, signals corresponding to the ^{15}N-labelled amino acid were observed selectively in each ^{1}H-^{15}N HSQC spectrum. Furthermore, without any inhibitors of transa-

minases, the ^{15}N-Pro specific labeling experiment for RbpA1, PHO4 basic helix-loop-helix domain, and ubiquitin gave no significant signals in ^{1}H-^{15}N HSQC spectra. This means that we can prepare selectively labeled proteins as for 17 amino acids other than Ala, Asp, Glu, without any change in the contents of the reaction mixture for the wheat-germ protein synthesis system, which also indicates that almost of all the enzymes participating in the metabolism of these 17 amino acids are inactivated or not included in the wheat germ extract.

On the other hand, in the cases of ^{15}N-Ala, ^{15}N-Asp, and ^{15}N-Glu selective labeling, NH signals corresponding to ^{15}N-Ala, ^{15}N-Asp, and ^{15}N-Glu were all observed in each ^{1}H-^{15}N HSQC spectrum (Figure 1). From this result, we speculated that two transaminases participating in the inter-conversion between Ala and Glu (alanine transaminase), and Glu and Asp (aspartate trasaminase) are active in the wheat germ extract (Figure 5). To examine this possibility, we at first synthesized RbpA1s in the presence of 7 mM β-chloro-L-alanine, which is known as an inhibitor of alanine transaminase, and checked the former hypothesis. As shown in Figure 2a, ^{15}N-Ala is utilized to synthesize RbpA1 without any transamination and, as shown in Figure 2b and c, signals corresponding to ^{15}N-Ala were completely absent, which indicates that the transamination reaction from Glu and Asp to Ala must be mediated mainly by alanine transaminase.

Next, we attempted to inhibit aspartate transaminase and checked the latter hypothesis. For this purpose, we used aminooxyacetate, which is known as an inhibitor of aspartate transaminase. As speculated, in the presence of aminooxyacetate, only signals corresponding to ^{15}N-Asp were observed in ^{1}H-^{15}N HSQC spectrum of RbpA1 and ubiquitin synthesized with the Asp-specific ^{15}N-labeled amino acid mixture. However, in the presence of only aminooxyacetate, the total amount of the synthesized RbpA1 was drastically decreased at about 30% with 0.4 mM aminooxyacetate. To overcome this problem, we used 0.4 mM aminooxyacetate and 2 mM β-chloro-L-alanine, simultaneously. In the presence of both inhibitors, almost the same amount of synthesized RbpA1 was obtained. Under these conditions, only ^{15}N-Asp signals were observed also. Furthermore, only ^{15}N-Glu signals were observed in the ^{1}H-^{15}N HSQC spectrum of RbpA1 synthesized with the ^{15}N-Glu selectively labeled amino acid mixture. These results indicate that in wheat germ extracts, the transamination reaction between Glu and Asp is mediated by aspartate transaminase, which is inhibited by 0.4 mM aminooxyacetate effectively. In the case of ubiquitin, even 1.0 mM of aminooxyacetate did not affect the amount of synthesized ubiquitin and this high concentration of aminooxyacetate also inhibited the metabolism of Ala and Glu effectively (Figures 3c–e). Though the reason why aminooxyacetate affect the amount of synthesized protein in some cases is unknown, one possible reason causing this difference is the optimum pH value for each protein synthesis. Then, we can use only aminooxyacetate at 1.0 mM to inhibit the metabolism among Ala, Glu, and Asp, if this concentration of AOA does not affect the protein synthesis. Alternately, we can use the combination of transaminase inhibitors, 2.0 mM of β-chloro-L-alanie and 0.4 mM of aminooxyacetate, if higher concentration of aminooxyacetate does affect the protein synthesis.

Furthermore, in the case of ubiquitin, weak Gln signals were observed in ^{1}H-^{15}N HSQC spectra for Glu selective labeling even if in the presence of 1.0 mM aminooxyacetate. This might be resulted by the weak glutamine-synthase activity in the wheat-germ extracts. This synthase activity must convert Glu to Gln during the long time synthesis of ubiquitin for 4 days and the accumulation of ^{15}N-labeled Gln in ^{15}N-Glu specific labeling experiment can not be overlooked. However, we can overcome this problem easily by using L-methionine sulfoximine as a glutamine synthase inhibitor. L-methionine sulfoximine did not affect the protein synthesis and completely inhibited the amino acid conversion from Glu to Gln (Figure 4). In spite of this, signals from NH_2 groups are observed in Figure 4. In this experiment, ^{1}H-^{15}N HSQC spectrum for selectively labeled ubiquitin was measured without any purification step. In the crude sample, many intrinsic proteins of wheat germ extracts were included and the total amount of these proteins is much larger than that of ubiquitin (Morita et al., 2003). Then, the observed NH_2 signals in Figure 4 are due to the $^{15}NH_2$ groups (natural abundance) of Gln and Asn residues constituting the intrinsic proteins of wheat germ extract.

For protein synthesis systems involving *E. coli*, many types of mutants or inhibitors have been used for selective labeling experiments (Muchmore et al., 1989; Kigawa et al., 1995; Waugh, 1996). In our wheat germ cell-free protein synthesis system, only two or three inhibitors are needed (no mutants are needed) to monitor ^{1}H-^{15}N HSQC spectra amino acid-specifically (Figure 5).

In summary, our results strongly indicate that our newly developed method for amino acid-specific monitoring in the ^{1}H-^{15}N HSQC spectra of proteins synthesized with the wheat germ cell-free protein synthesis system is one of the best ways for precise analysis of both protein structures, and the inter-molecular interactions between the proteins of interest and some ligands, in a high-throughput manner.

Acknowledgement

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