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Recommendations for the Selective Labeling of [^{15}N]-Labeled Amino Acids without Using Auxotrophic Strains

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Abstract: The strategy to incorporate [^{15}N]-labeled amino acids were discussed. Instead of using specific auxotrophic strains for selective labeling, the prototrophic strain, BL21(DE3), was used with a plasmid, pLysS, and found to be very effective for several amino acids including alanine, lysine, leucine, and threonine. Isoleucine, valine, glutamine, and tyrosine were also found to be effective despite some diffusion into other amino acids. Interesting result was obtained when [^{15}N]-labeled glycine was tried: only glycines were labeled when amino acid mixture was added in the growth medium, and serines were co-labeled when amino acids were omitted. These results can be used as a guideline when selective labeling strategy is considered, and when the resulting data are interpreted.

INTRODUCTION

Selective labeling strategy has been regarded as an efficient way to simplify the complex NMR spectra. With this strategy, we can make only the desired signals appear ([^{13}C]- or [^{15}N]-labeling[1]), or undesired ones disappear ([^{2}H]-labeling[2]) in the normal NMR spectrum. This facilitates the analysis of the large molecules[3] or macromolecular complexes[4] as well as resonance assignments. However, the selective labeling strategy has been relying on the auxotrophic strains to prevent the input labeled amino acids from dilution, which posed formidable obstacles because some auxotrophic strains are not suitable for overproduction of proteins. Furthermore, the "perfect" auxotrophic strains are very difficult to obtain because the anabolism as well as the catabolism of a specific amino acid should be knocked out. In other words, it must not make nor utilize the desired amino acid. In this article, I'll talk about using the prototrophic strain, BL21(DE3), to prepare selectively labeled samples instead of using the problematic auxotrophic strains.

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MATERIALS AND METHODS

The heterocyst ferredoxin from *Anabaena* 7120 was overexpressed in *Escherichia coli*, refolded *in vitro*, and purified as described in Jacobson et al.[5], and Chae et al.[6] The prototrophic strain, BL21(DE3) containing pLysS, was used in the labeling process, either uniform or selective. For the uniform ^{15}N labeling, 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, and 1 g of [UL ^{15}N, 99%]NH_4Cl was dissolved in one liter of distilled water. Upon inoculating this medium, 4 g of glucose was added with proper antibiotics (in the present case, 50 mg kanamycin and 34 mg chloramphenicol were added in one liter culture).100 ml of the minimal medium culture which was grown overnight was used as the inoculum.

For selective labeling, a medium containing the appropriate unlabeled amino acid mixture was prepared[7]: to one liter of distilled water, 0.50 g alanine, 0.40 g arginine, 0.40 g aspartic acid, 0.05 g cysteine, 0.40 g glutamine, 0.65 g glutamic acid, 0.55 g glycine, 0.10 g histidine 0.23 g isoleucine, 0.23 g leucine, 0.42 g lysine-HCl, 0.25 g methionine, 0.13 g phenylalanine, 0.10 g proline, 2.10 g serine, 0.23 g threonine, 0.17 g tyrosine, and 0.23 g valine. In addition to this amino acid mixture, 0.50 g adenine, 0.50 g uracil, 0.20 g thymine, 0.20 g cytosine were added. Guanosine was omitted from the original recipe. Two recipes were used for other components of the medium: one solution contained 1.50 g sodium acetate, 1.50 g succinic acid, 0.50 g NH_4Cl, 0.85 g NaOH, and 10.50 g K_2HPO_4; the other was M9 solution (6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, and 0.5 g NaCl). Either solution yielded the same amount of selectively labeled heterycyst ferredoxin, although the medium containing M9 solution showed faster growth of the cells. Upon inoculation, 1 ml of 2 M $MgSO_4$, 1 ml of 0.2 M $CaCl_2$, 100 μl of 20 mg/ml thiamine, 100 μl of 50 mg/ml $FeCl_3$, 1 ml of 50 mM $ZnSO_4$ were added along with 4 g of glucose and proper antibiotics. The amino acids corresponding to the residues to be labeled were initially omitted from the amino acid mixture. As described above, 100 ml overnight culture was used as the inoculum for one liter culture. Upon induction by 200 mg of IPTG, 50 – 200 mg of the desired amino acid labeled with [^{15}N, 90-99%] was added. The subsequent steps were the same as described above. The sites labeled were determined by NMR spectroscopy. NMR Spectra were collected on a Bruker AM600 spectrometer. The HMQC pulse sequence[8] was used to collect data.

RESULTS AND DISCUSSION

(1) Aspartate and glutamate residues did not get labeled selectively

In Figure 1, the HMQC spectrum of the uniformly [^{15}N] labeled sample. It shows good dispersion indicating that the β-strands are the major secondary structure in this molecule. In Figure 2, the samples prepared from the [^{15}N] labeled Asp and Glu show no significant difference from the uniformly labeled sample if stronger signals are considered. The number

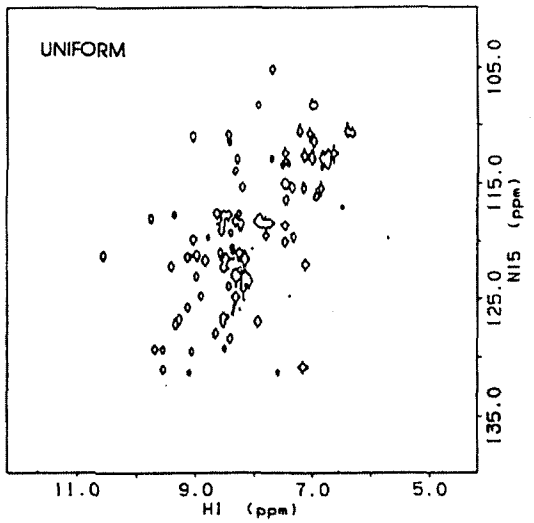

Fig. 1. HMQC spectrum of [U, ^{15}N] heterocyst ferredoxin (reproduced from ref. 9)

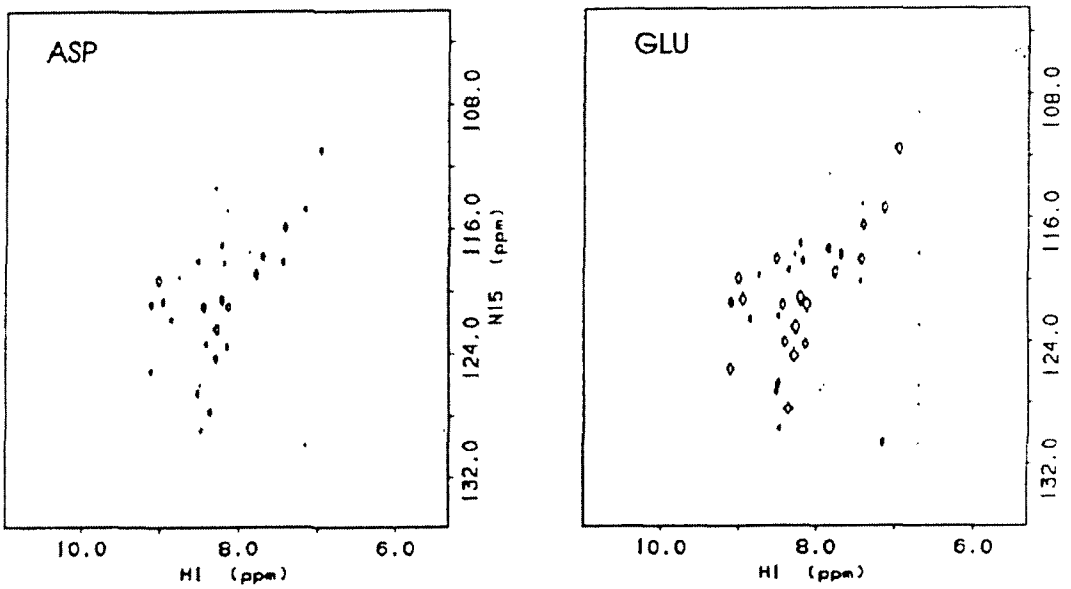

Fig. 2. HMQC spectra of [^{15}N]Asp- and Glu-labeled heterocyst ferredoxin (reproduced from ref. 9)

of resonance signals is too large, and the intensities of the signals are quite uniform throughout the spectrum. This result was expected because Asp and Glu are located at the center of the metabolic pathway. In other words, Asp and Glu can be made and utilized within the cells very efficiently, resulting in the dilution and diffusion of the input labels.

(2) Alanine, lysine, leucine, and threonine residues got labeled selectively without diffusion of the input labeled materials.

As can be seen in Figure 3, Ala, Lys, Leu, and Thr residues were labeled cleanly without other detectable peaks. The interesting result is about the labeling with [^{15}N]-Alanine. The signals from the Ala residues are less intense than those from Lys, Leu, or Thr. Considering the amounts of the input labeled amino acids are the same, this is probably either because the unlabeled alanine molecules were produced inside the host to the amount comparable to the input, leading to the dilution of the input material, or because the labeled alanine molecules were not efficiently transported into the host, which also results in the dilution of the input labels.

(3) Isoleucine, glutamine, valine, and tyrosine residues were labeled selectively with minor diffusion of the input.

Unlike the amino acids in the previous paragraph, Ile, Gln, Val, and Tyr residues showed the diffusion of the input labels. The degree of diffusion was not severe as compared to the Asp and Glu residues. The inclusion of isoleucine in this category was somewhat surprising because Ile is located near the center of the metabolism. The input labels of Gln, Val, and Tyr diffused, but into the specific amino acids. For example, Gln preferentially diffused into Glu, Val into Ala, and Tyr into Phe. From this result, the synthesis of Ala and Phe is downstream to that of Val and Tyr. The conversion of Val into Ala seems efficient, and this could be the partial reason why the alanine label got diluted as mentioned above. In some cases, this kind of minor diffusion can be advantageous because two kinds of residues get labeled simultaneously with the varying degree, and the signals can be easily discriminated by the peak intensity.

(4) Glycine residues can be labeled in two ways.

Glycine was the most interesting amino acid because there were two ways to label it. If the amino acid mixture mentioned in the Materials and Methods was used, only Gly residues got labeled. If the M9 solution was used instead of the amino acid mixture, serine residues also got labeled. This is a very encouraging result because [^{15}N]Ser is very expensive, and yet very hard to label without the help of the auxotrophic strain because Ser is located near the center of the metabolic pathway. When BL21(DE3) was used as a host, input serine labels diffused very fast primarily into the amide nitrogen atoms of the side chains (Gln or Asn) without labeling those of Ser (data not shown). Since Gly can be used to label Ser, this is a good and cheap way to label Ser, and the serine residues can be easily

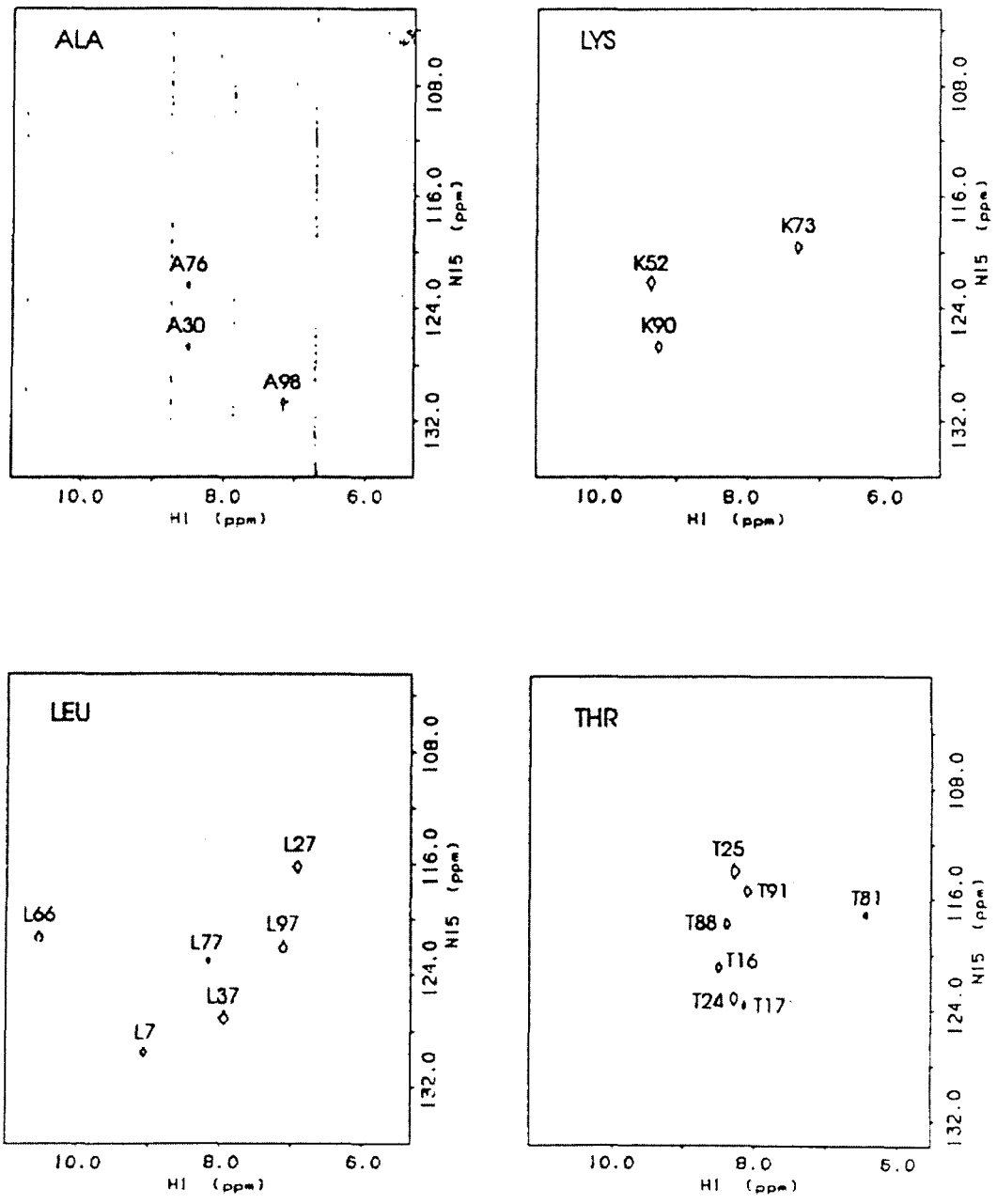

Fig. 3. HMQC spectra of [^{15}N]Ala-, Lys-, Leu-, and Thr-labeled heterocyst ferredoxin (reproduced from ref. 9)

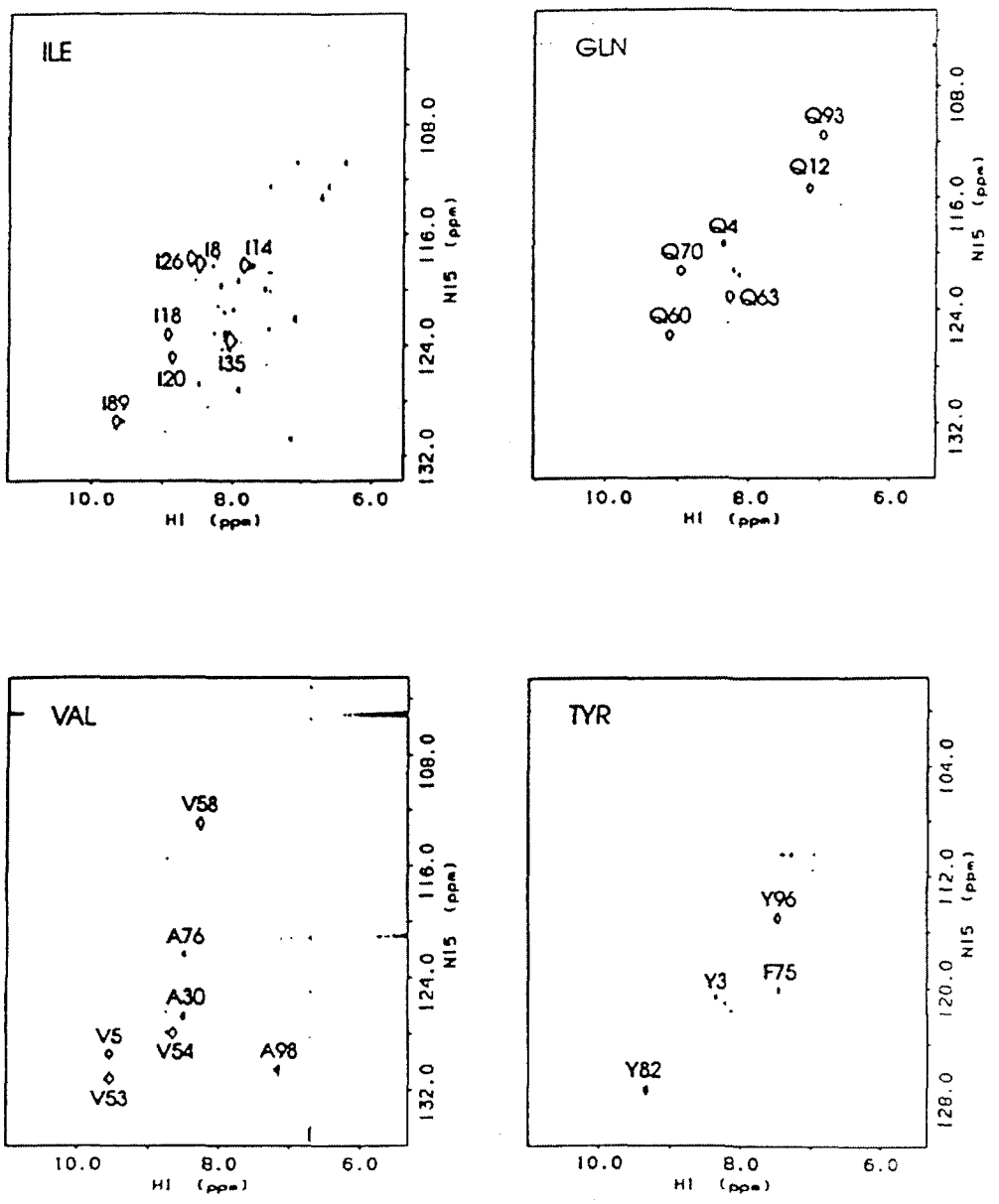

Fig. 4. HMQC spectra of [^{15}N]Ile-, Gln-, Val-, Tyr-labeled heterocyst ferredoxin (reproduced from ref. 9)

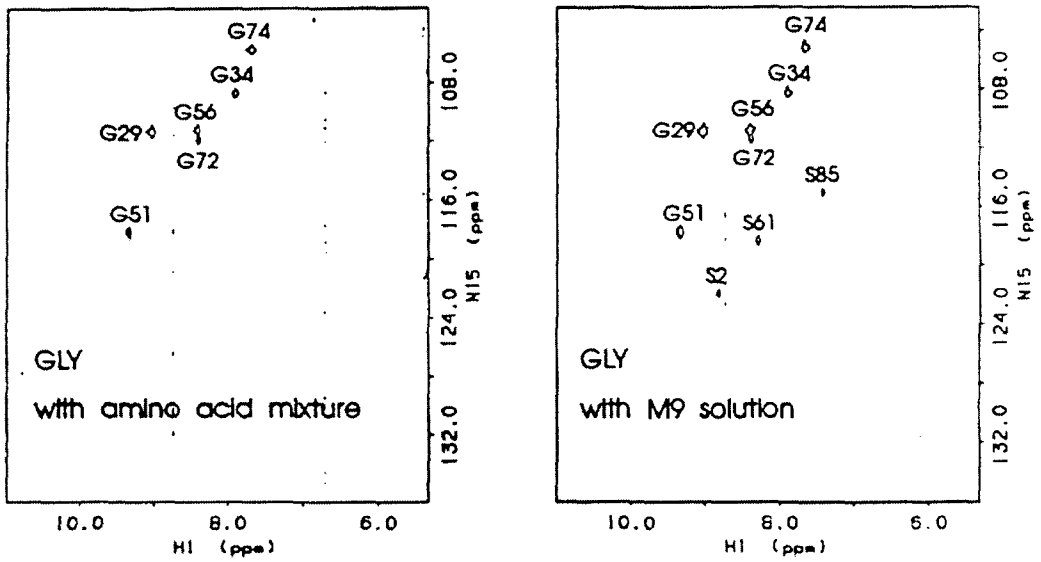

Fig. 5. HMQC spectra of [^{15}N]Gly-labeled heterocyst ferredoxin with two different recipes (reproduced from ref. 9)

discriminated from the glycine residues if the two HMQC spectra of Gly only- and Gly and Ser-labeled samples are compared.

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