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A Practical Method for Uniform Isotopic Labeling of Recombinant Proteins in Mammalian Cells

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ABSTRACT: A method to obtain uniformly isotopically labeled (^{15}N and $^{15}N/^{13}C$) protein from mammalian cells is described. The method involves preparation of isotopically labeled media consisting of amino acids isolated from bacterial and algal extracts supplemented with cysteine and enzymatically synthesized glutamine. The approach is demonstrated by producing ^{15}N-labeled and $^{15}N/^{13}C$-labeled urokinase from Sp2/0 cells and successfully growing Chinese hamster ovary (CHO) cells on the labeled media. Thus, using the procedures described, isotopically labeled proteins that have been expressed in mammalian cells can be prepared, allowing them to be studied by heteronuclear multidimensional NMR techniques.

Heteronuclear multidimensional NMR[1] has become a powerful method for determining the three-dimensional structures of proteins in solution (Clore & Gronenborn, 1991; Stockman et al., 1992; Clubb et al., 1991; Neri et al., 1991; Driscoll et al., 1990; Ikura et al., 1990; Torchia et al., 1989). This approach requires isotopically labeled protein, typically uniformly enriched (>95%) with ^{15}N and double enriched with ^{15}N and ^{13}C. The isotopically labeled protein is usually obtained from bacterial cells in which the protein is overexpressed that are grown on ^{15}N- or $^{15}N/^{13}C$-enriched media (Muchmore et al., 1989). However, many proteins of interest, especially eukaryotic proteins, cannot be obtained from bacteria in their properly folded form. Although these proteins may be expressed in mammalian cells (Levinson, 1990) to produce soluble, properly folded proteins for NMR, mammalian cells will not grow on isotopically enriched media typically used to label proteins from bacteria. Mammalian cells require amino acids, vitamins, cofactors, and in most cases serum and are more sensitive to toxic substances than bacteria (Thomas, 1990; Eagle, 1955).

In this paper, we describe a practical, cost-effective approach for uniformly ^{15}N- and $^{15}N/^{13}C$-labeling proteins in mammalian cells, allowing the three-dimensional structures for these proteins to be studied using powerful heteronuclear three- and four-dimensional NMR techniques (Fesik & Zuiderweg, 1988; Kay et al., 1990; Fesik & Zuiderweg, 1990; Clore & Gronenborn, 1991). We demonstrate the method by preparing isotopically labeled urokinase which has been expressed in Sp2/0 cells (Lo & Gillies, 1991). Although urokinase itself is too large for detailed NMR studies, this protein can be proteolytically cleaved (Mazar et al., 1992) into an amino-terminal fragment (ATF) (14 kDa) composed of a kringle and a growth factor domain that binds the urokinase receptor (Appella et al., 1987) and a serine protease domain (30 kDa) that cleaves plasminogen to plasmin. The structures of both fragments are of interest due to their apparent role in metastatic tissue invasion (Danø et al., 1985; Mignatti et al., 1986; Hearing et al., 1988; Boyd et al., 1988; Ossowski, 1988; Testa & Quigley, 1990), fibrinolysis (Zamarron et al., 1984; Gurewich et al., 1984; Pannell & Gurewich, 1986), chemotaxis (Gudewicz & Gilboa, 1987; Fibbi et al., 1988), and growth factor activity (Rabbani et al., 1992).

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[1] Abbreviations: NMR, nuclear magnetic resonance; ATF, amino-terminal fragment of urokinase; TLC, thin-layer chromatography; CHO, Chinese hamster ovary cells; MAb, monoclonal antibody; PBS, phosphate-buffered saline; HSQC, heteronuclear single-quantum correlation; 1D, one dimensional.

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MATERIALS AND METHODS

Extraction of Protein Fraction. ^{15}N-Labeled amino acids were obtained from bacteria while $^{15}N/^{13}C$-labeled amino acids were isolated from lyophilized algae (Cambridge Isotopes). *Escherichia coli* cultures (JM109) were grown to a high density by slow feeding with glucose in a 10-L fermenter containing 20 g of [^{15}N]ammonium chloride to produce 65 g of lyophilized cells. Treatment of the lyophilized biomass from either algal or bacterial sources to isolate the protein fraction was essentially identical, following the method of Putter et al. (1969). Briefly, it involves extraction of the biomass with 10% trichloroacetic acid at 70 °C to break apart the cells and remove nucleic acids, followed by ethanol/ether and ether extractions to remove lipids, chlorophyll, and small organic molecules. In a typical preparation 20 g of lyophilized cells yielded between 8 and 12 g of protein.

Enzymatic Hydrolysis. Enzymatic digestion of the protein fraction was accomplished by dissolving approximately 2.8 g of the algal protein extract in 200 mL of a 6 M urea solution. A small amount of material that remained undissolved was removed by centrifugation. This protein solution was then dialyzed exhaustively against distilled water (3500 molecular weight cutoff tubing). The dialyzed solution was adjusted to neutral pH and made 5 mM in calcium chloride. Pronase (Boehringer-Mannheim) was added (160 μg/mL), and the solution was incubated for 12 h at 37 °C. In order to kill the protease activity, the solution was autoclaved for 30 min at 126 °C and then lyophilized. The final yield was approximately 1.5 g. Amino acid analysis showed that approximately one-third of the amino acids were in the free form while the remainder were presumably in the form of small peptides.

Endotoxins were removed from the amino acid mixture using the Pyrofree III microporous affinity matrix (Alerchek) by following the manufacturer's instructions.

Acid Hydrolysis and Purification. Ten grams of the isolated protein fraction was hydrolyzed under vacuum in a sealed 500-mL ampule with 150 mL of 4 M methanesulfonic acid at 115 °C for 22 h (Simpson et al., 1976). Two grams of tryptamine and 2 g of imidazole were added as suicide bases to protect tryptophan and histidine during hydrolysis (LeMaster & Richards, 1982). After hydrolysis, purification of the amino acids was accomplished using a modification of the procedure of LeMaster and Richards (1982). The changes simplify the separation and improve the flow rate at the expense of resolution which is not a concern, since all of the amino acids are combined in the cell culture media. The procedure begins by diluting the hydrolysis mixture to 4 L with water and filtering the solution to remove any insoluble particles. The amino acids in the dilute methanesulfonic acid were loaded onto a cation-exchange column (5-cm diameter × 20 cm) (AG50W-X8, 200–400 mesh, Bio-Rad) in the hydrogen form. After loading, the column was washed with 2 L of water and then eluted with 100 mM piperidine. When elution with piperidine begins, a visible band containing the amino acids forms and moves down the column leaving a very dark band at the top. The eluant was collected, and the fractions containing amino acids were identified by spotting 5 μL on filter paper and spraying with 0.2% ninhydrin in ethanol.

Ninhydrin-positive fractions were pooled and rotoevaporated to dryness. The dry amino acids were again dissolved in water, the pH was adjusted to 11.5 with sodium hydroxide (above the pK_a of ammonia and piperidine), and the fractions were rotoevaporated to dryness. This step was repeated until the pH stabilized, indicating that no more ammonia or piperidine was being removed. The total yield of amino acids from 20 g of lyophilized cells was typically about 8 g.

Amino acids isolated at this point were yellow, indicating the presence of impurities. Although these amino acids were able to support mammalian cell growth, growth rates were improved through further purification by passing the amino acid mixture through a 500 molecular weight cutoff membrane using an Amicon 400-mL stirred cell. This step removed much of the yellow color from the amino acids and most of the unidentified toxins. After passage through the membrane, the amino acids were lyophilized and stored at 4 °C until needed.

The amino acid composition of each preparation was determined by amino acid analysis using a Beckman 6300 instrument.

Enzymatic Synthesis of Labeled Glutamine. One and a half grams of glutamate (^{15}N or $^{15}N/^{13}C$, Cambridge Isotopes) was added to a flask with 0.58 g of $^{15}NH_4Cl$ (Cambridge Isotopes), 9 g of ATP (Sigma), and 1.35 g of $MgCl_2$. The pH was adjusted to 7.6, and 500 units of glutamine synthatase (Sigma) was added. Gentamicin, 50 μg/mL, was added to help prevent bacterial growth during incubation. The reaction was carried out after filter sterilization (Nalgen, cellulose acetate membrane) in the 1-L receiving flask by incubating the flask at 37 °C for several days. During the long incubation, necessary to conserve the enzyme, it was extremely important to keep the flask rigorously sterile. Any bacterial growth would be toxic to the mammalian cells. Completeness of the reaction was measured by spotting 2 μL on a silica TLC plate (Merck) that was developed with a 2-propanol/formic acid/water (40:2:10) solution. After being dried the TLC plate was sprayed with 0.2% ninhydrin in ethanol (R_f values: NH_4^+ 0.54, Glu 0.48, Gln 0.37).

After 4 days the reaction was essentially complete. The reaction mixture was then purified using an anion-exchange column, AG1-X8 (Bio-Rad) in the chloride form (Prusiner & Milner, 1970). The pH of the reaction mixture was first adjusted to 6.0 and then passed through the column which retained ATP and ADP which are necessary to remove since they can be toxic to mammalian cells if present in large amounts (Eagle et al., 1956). The glutamine, which does not bind to the column, was collected and lyophilized. The other components of the reaction mixture which pass through the column with glutamine are common components of cell culture media.

Cell Culture. Heat-treated, dialyzed serum was prepared by heating 1 L of fetal bovine serum (Gibco) at 65 °C for 60 min. After heat treatment the serum was placed in dialysis tubing (1000 molecular weight cutoff, SpectraPor) and equilibrated against three changes of 20 L of phosphate-buffered saline (Gibco).

To test the growth response of our cell line to varying concentrations of amino acids and any toxicity of our amino acid sources, cell cultures were carried out in 100-mL spinner flasks (Belco). Our amino acids were added to hybridoma-SFM media prepared without amino acids (Gibco) and supplemented with 5% heat-treated, dialyzed serum. Sp2/0 cells were planted at 0.1×10^6 viable cells/mL. Cell counts were done at 3 and 5 days.

Large-scale growths to prepare NMR samples were done in 1-L spinner flasks. The media (Gibco hybridoma-SFM without amino acids) were supplemented with 5% dialyzed, heat-treated serum, 2.5 μg/mL methotrexate, and 5 μM aprotinin (Sigma). Labeled amino acids (1.2 g) were added with 500 mg of synthesized labeled glutamine and 40 mg of [^{15}N]cysteine (Cambridge Isotopes). Spinner flasks were

planted at 0.2×10^6 viable cells/mL. Large growths also contained gentamicin (12.5 μg/mL), penicillin (100 units/mL) and streptomycin (100 μg/mL). Cell growth has allowed to proceed until nutrients were exhausted and cell viability fell below 10%. The total cell density varied but was typically $(1.0–2.0) \times 10^6$ cells/mL when harvested.

Chinese hamster ovary (CHO) cells were grown attached in 75-cm^2 flasks. Cells were split 1:4 at each passage level in F12 media plus 10% heat-treated, dialyzed serum. After attachment, cells were grown for 7 days on different media to compare cell growth.

Urokinase Preparation and Activity Assays. Urokinase was purified from the harvested media using immunoaffinity chromatography. A MAb which is directed against the C-terminal portion of the kringle domain of urokinase was immobilized using CNBr–Sepharose (Sigma) (Mazar et al., 1992). Typically, 300 mg of MAb was immobilized to CNBr–Sepharose (45 mL) with >99% efficiency. A column was then prepared using the immobilized MAb–Sepharose and equilibrated with phosphate-buffered saline (PBS) (Gibco). Harvested media (0.75–1.0 L) were loaded at a flow rate of 0.25–0.5 mL/min, and the column was washed with PBS until no protein UV spectrum could be observed in the pass through. Bound protein was eluted with 0.1 M sodium acetate, pH 4.2, pooled, concentrated, and stored at –70 °C. ATF and the serine protease domain were purified from the eluant as previously described (Mazar et al., 1992).

The NMR sample of the purified ATF fragment (0.5 mM) was prepared by dissolving the protein in 0.5 mL of sodium acetate-d_3 buffer (pH 4.5, 90% H_2O/10% D_2O).

NMR Spectroscopy. All NMR spectra of [U-^{15}N]ATF were acquired on either a Bruker AMX600 or a AMX500 spectrometer at 25 °C with the ^{1}H carrier set on the water frequency. The ^{1}H/^{15}N HSQC spectrum (Bodenhausen & Ruben, 1980) was collected with 233 (t_1) × 2048 (t_2) complex points in a total acquisition time of 14 h. The ^{15}N-coupled and ^{15}N-decoupled spectra along with the ^{15}N-isotope-filtered 1D NMR spectrum (Otting et al., 1986) were collected with the spectral width of 10 000 Hz. The spectra were processed and analyzed using in-house-written software on Silicon Graphics computers.

RESULTS AND DISCUSSION

In principle, an isotropically labeled medium could be prepared by combining the necessary vitamins and inorganic salts with amino acids that have been isotopically labeled and individually purified in the ratio of typical mammalian cell media (Table I). However, some of the isotopically labeled amino acids are not commercially available, and most are extremely expensive. A more practical approach is to use an isotopically labeled amino acid mixture isolated from bacteria or algae grown on readily available and relatively inexpensive $^{15}NH_4Cl$ and ^{13}C-labeled glucose or $^{13}CO_2$.

In an initial attempt to prepare isotopically labeled amino acids for mammalian cell growth, algal protein was enzymatically hydrolyzed with Pronase. This approach was taken in order to preserve glutamine, asparagine, cysteine, and tryptophan which are largely destroyed by acid hydrolysis. Although the yield and relative proportions of the amino acids prepared by this procedure were comparable to those present in the commercial media (Table I), media prepared with these amino acids were unable to support cell growth. Cells planted in media containing amino acids from proteolytic digestions did not divide and died after a few days, even when supplemented with the amino acids from the commercial media. These results suggested that cell death was caused by toxic impurities in the amino acid isolate. Therefore, several attempts were made to further purify the isolated mixture of amino acids. Although passage of the amino acids through a 500 molecular weight cutoff membrane recovered much of the faint color caused by impurities, the amino acid mixture was still fatal to cell culture as indicated in Figure 1. Removal of the endotoxins typically found in algal cell extracts that are known to be toxic to mammalian cells with an endotoxin affinity matrix also failed to provide amino acids able to support cell growth (Figure 1).

Table I: Amino Acid Composition of Different Media Preparations[a]

	commercial media	algal enzymatic digestion	bacterial acid hydrolysis	algal acid hydrolysis
Ala	30	158	167	215
Arg	178	181	180	130
Asn	100	*b*	0	0
Asp	90	205	297	195
Cys	83	215	0	0
Gln	600	*b*	0	0
Glu	20	260	331	290
Gly	130	112	118	130
His	46	38	56	55
Ile	147	105	128	100
Leu	160	188	247	245
Lys	160	150	180	270
Met	45	50	69	40
Phe	86	132	121	165
Pro	247	150	118	135
Ser	100	75	111	95
Thr	100	125	128	120
Trp	28	60	trace	trace
Tyr	83	125	129	160
Val	117	175	165	150

[a] All concentrations in milligrams per liter. [b] For enzymatic digestion, values of Asp and Glu include Asp and Gln.

Due to the lack of success with the amino acid mixtures prepared by enzymatic hydrolysis, we resorted to acid hydrolysis of bacterial or algal protein and purification via ion-exchange chromatography. The procedure that we developed results in the purification of the amino acid mixture without resolution of any individual amino acids. It is relatively straightforward to implement and very reproducible. As shown in Table I, the typical yields of amino acids from either bacteria or algae are similar to each other. Indeed, both bacteria and algae are a convenient and inexpensive source of ^{15}N-labeled biomass, since $^{15}NH_4Cl$ is the only labeled nutrient that is required. However, for labeling with ^{15}N/^{13}C, algae are preferred, since with algae $^{13}CO_2$ is used as the only carbon nutrient, which is much less expensive than ^{13}C-labeled glucose (the typical carbon source in bacteria).

Unlike the enzymatically prepared amino acid mixture, media prepared from the acid hydrolysate supplemented with the amino acids that are destroyed during hydrolysis were able to sustain mammalian cell growth (Figure 1). In order to improve the growth, additional purification of the amino acid mixture was attempted. Of the procedures tried, passage of the amino acids through a 500 molecular weight cutoff membrane improved cell growth the most. As shown in Figure 1, both ^{15}N- and ^{15}N/^{13}C-labeled media supported cell growth to near the level of commercial media.

With a purification procedure in hand for preparing an amino acid mixture that was nontoxic to mammalian cells, the next step was to determine which of the supplemented amino acids were required and what was the minimum amount necessary for cell growth. For these tests, mammalian cells

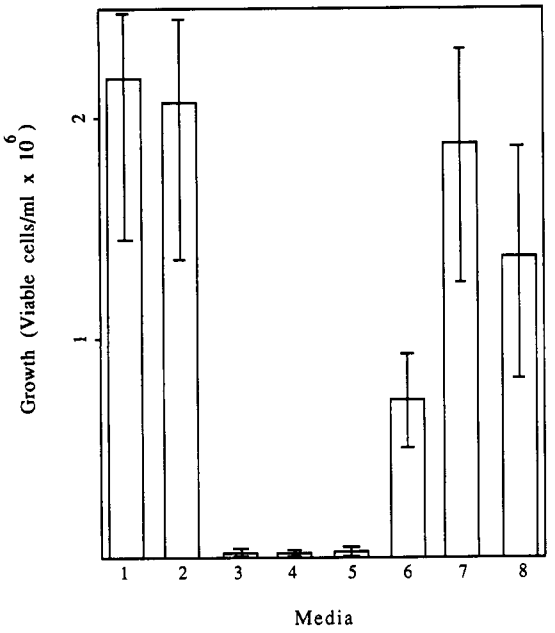

FIGURE 1: Growth response of Sp2/0 cells as a function of different amino acid mixtures. The basal media for all cultures were Gibco hybridoma-SFM without amino acids supplemented with 5% heat-treated, dialyzed serum. Amino acids from the acid hydrolysis were supplemented with amino acids (Gln, Cys, and Trp) destroyed during hydrolysis. Media: (1) unlabeled, purified amino acids at the commercial concentrations (Table I); (2) unlabeled, purified amino acids at the algal hydrolysis concentrations; (3) amino acids from algal enzymatic digestion; (4) amino acids from enzymatic digestion passed through Amicon with a 500 molecular weight cutoff membrane; (5) amino acids from enzymatic digestion treated with endotoxin affinity resin; (6) uniformly ^{15}N-labeled amino acids from bacterial cells prepared by acid hydrolysis; (7) ^{15}N-labeled amino acids from bacterial cells prepared by acid hydrolysis that were passed through a 500 molecular weight cutoff membrane; (8) U-$^{15}N/^{13}C$-labeled amino acids from algal cells prepared by acid hydrolysis that were passed through a 500 molecular weight cutoff membrane. Error bars are based on the results from duplicate cultures.

were grown on media prepared using concentrations of amino acids obtained from algal cell acid hydrolysate (Table I) and varying amounts of the supplemented amino acids. As shown in Figure 2, asparagine was not required. In contrast, glutamine and cysteine were found to be necessary for growth of Sp2/0 cells. The concentration dependence of these amino acids is shown in Figure 2. These results are consistent with earlier studies on other cell lines (Eagle, 1959). Figure 2 also depicts the dependence of cell growth on varying amounts of tryptophan. As can be seen, only very small amounts of this amino acid are needed. In fact, the trace amount of tryptophan obtained in the acid hydrolysis (Table I) was found to be sufficient. Thus, it was necessary to supplement the hydrolysis mixture with only glutamine and cysteine.

Relatively large amounts of glutamine were required for cell growth, which was expected due to the importance of glutamine in the metabolic pathway for several amino acids and nucleic acids. Unfortunately, [U-^{15}N]Gln is relatively expensive and [U-$^{15}N/^{13}C$]Gln is not commercially available. Therefore, we investigated methods for preparing isotopically labeled glutamine. After trying several methods, we enzymatically prepared both ^{15}N- and $^{15}N/^{13}C$-labeled glutamine in high yields using glutamine synthetase from glutamate, ^{15}N-labeled ammonium chloride, and ATP. Both ^{15}N- and $^{15}N/^{13}C$-labeled glutamates are readily available and comparatively inexpensive. Cysteine was supplemented using [^{15}N]cysteine for the ^{15}N- and $^{15}N/^{13}C$-labeled growths. Doubly labeled cysteine is not commercially available.

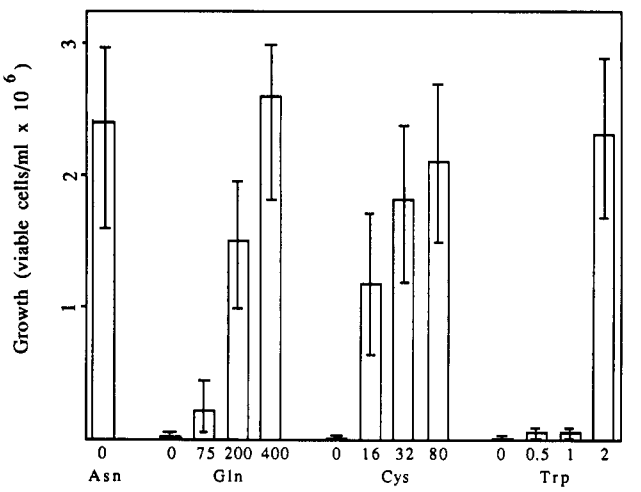

FIGURE 2: Growth response of Sp2/0 cells as a function of varying amino acid concentrations. The basal media were Gibco hybridoma-SFM without amino acids plus 5% heat-treated, dialyzed serum. Unlabeled amino acids were added at the concentration obtained from acid hydrolysis plus 600 mg/L Gln, 80 mg/L Cys, and 20 mg/L Trp except as indicated in the figure. Cells were planted at 0.1×10^6 cells/mL and counted on the fifth day. Error bars are based on the results from duplicate cultures.

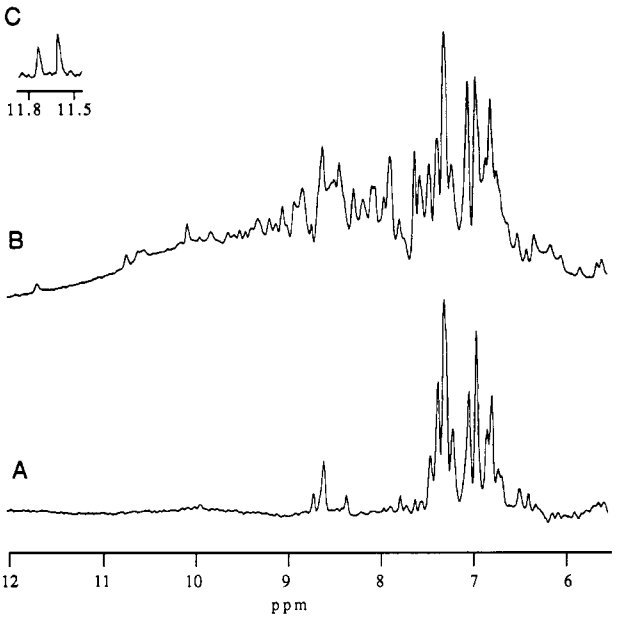

FIGURE 3: Downfield region of a (A) ^{15}N-isotope-filtered proton spectrum (600 MHz) and (B) ^{15}N-decoupled proton spectrum of [U-^{15}N]ATF. (C) ^{15}N-Coupled proton spectrum of [U-^{15}N]ATF showing an isolated resonance at 11.6 ppm.

Supplementation of media with serum typically improves growth of mammalian cells, but in our case it was not a requirement. A potential disadvantage of including serum in the media was the possibility of diluting the isotope label with unlabeled amino acids found in the serum. In order to remove these amino acids, the serum was dialyzed against phosphate-buffered saline. The use of dialyzed serum did not affect the isotope enrichment of our protein as evidenced from the NMR spectra of [U-^{15}N]ATF shown in Figure 3. Comparison of a ^{15}N-isotope-filtered NMR spectrum in which the signals corresponding to the labeled amides have been suppressed (Figure 3A) with a ^{15}N-decoupled spectrum (Figure 3B) indicates that the sample is highly ^{15}N-enriched. (The signals at 8.5–8.8 ppm correspond to the eight His residues of ATF.) Another demonstration of the high level of enrichment is shown in a portion of the ^{15}N-coupled spectrum showing an isolated

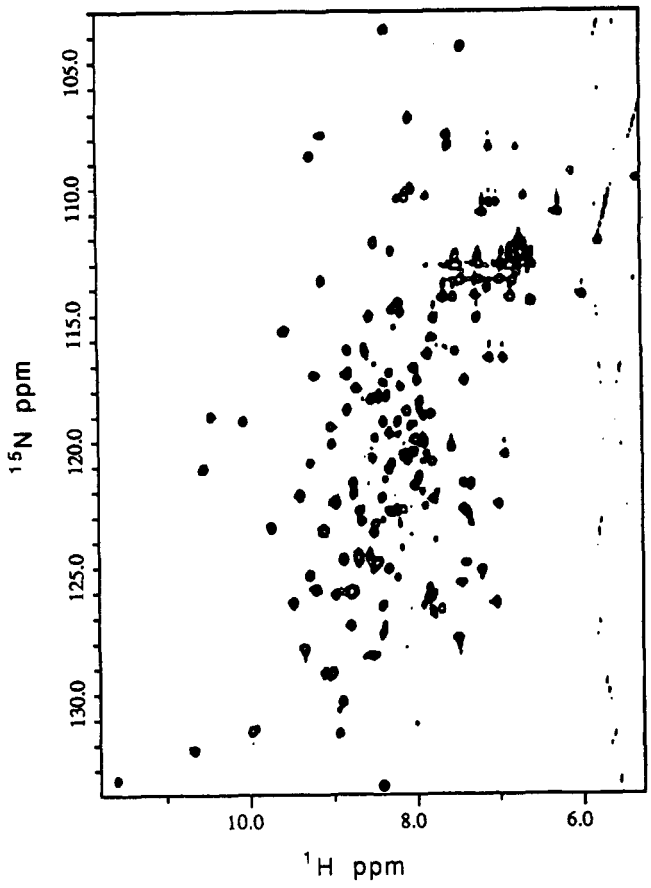

FIGURE 4: A complete $^{1}H/^{15}N$ HSQC spectrum (500 MHz) of [U-^{15}N]ATF.

peak (Figure 3C). Any unlabeled protein would be detected in this spectrum by a peak appearing between the doublet. On the basis of the relative intensities of the signals corresponding to the labeled and unlabeled protein, greater than 95% is labeled at this site.

From mammalian cells grown on media prepared by the procedures described above, a sufficient quantity of labeled urokinase was prepared for heteronuclear multidimensional NMR studies. From a 1-L growth, 30 mg of urokinase was obtained. Figure 4 depicts a portion of a $^{1}H/^{15}N$ HSQC spectrum of [U-^{15}N]ATF prepared from [U-^{15}N]urokinase. The number of cross peaks that appear in the spectrum is consistent with the expected number of signals. We have also prepared [U-$^{15}N/^{13}C$]ATF and are currently in the process of assigning the resonances and determining the three-dimensional structure of this protein as well as the other urokinase cleavage product—the serine protease domain.

In conclusion, we have developed a practical, cost-effective approach for isotopically labeling proteins with both ^{15}N and $^{15}N/^{13}C$ that are expressed in mammalian cells. The cost of 1 L of ^{15}N media was under one thousand dollars and was approximately four thousand dollars for $^{15}N/^{13}C$ media. The approach was demonstrated by isotopically labeling urokinase that was overexpressed in Sp2/0 cells. Recently, we have tested the generality of the method by attempting to grow CHO cells in the same isotopically labeled media. CHO cells, which have been used to express many proteins of biological interest, grew to an equivalent cell density in the unlabeled or labeled media. Thus, many isotopically labeled proteins could be prepared from mammalian cells grown on isotopically labeled media that have been prepared as described here which will allow the three-dimensional structure of these proteins to be determined using recently developed heteronuclear multidimensional NMR techniques.

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