Using Stable-Isotope-Labeled Proteins for Hydrogen Exchange Studies in Complex Mixtures

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The use of mass spectrometry to measure hydrogen exchange rates for individual proteins in complex mixtures is described. Incorporation of stable-isotope-labeled (SIL) amino acids into a protein of interest during overexpression in bacteria produced distinctive isotope patterns in mass spectra of peptic peptides from the labeled protein. The isotope pattern was used as a signature for peptides originating from the SIL protein. In addition, stable-isotope labeling simplified identification of the peptic peptides by providing partial amino acid composition information. Despite the complex isotope patterns associated with SIL peptides, hydrogen exchange rates could still be measured for peptides from SIL protein and were found to be the same as exchange rates for unlabeled protein. Hydrogen exchange in a single protein of interest was measured in a complex mixture of proteins, a bacterial cell lysate. This methodology, which includes easy recognition of peptic peptides from the protein(s) of interest during hydrogen exchange studies in heterogeneous systems, will permit analysis of structural properties and dynamics of large protein complexes and complex protein systems.

A complete understanding of large proteins and protein complexes requires knowledge about their structural nature and dynamics. To accomplish this, methods are needed that are capable of discerning subtle changes in the structure and dynamics of a specific protein within complex, heterogeneous systems. To make one protein distinguishable from other proteins, it can be tagged with stable isotopes. Stable isotopes allow the protein of interest to be recognized with various biophysical techniques without affecting its structural properties and functions.

The incorporation of stable isotopes such as ^{13}C, ^{15}N, ^2H, and ^{18}O into biomolecules has been invaluable for quantitation and for the analyses of metabolism, protein structure, and protein dynamics. Stable isotopes can be incorporated into molecules during chemical or biological synthesis, during enzymatic reactions, or through ^2H (deuterium) exchange of labile hydrogens

in solution. Molecules labeled with stable isotopes have been used as metabolic tracers,[1] in the NMR characterization of proteins,[2,3] and in peptide mapping with mass spectrometry (MS).[4,5] Recently, stable isotopes have been used to characterize complex protein mixtures such as whole proteomes[6−8] and to obtain additional sequence information during MS analysis.[9,10]

When a protein is placed into a solution of D_2O, labile hydrogens in the protein are replaced with deuterium at varying rates depending on hydrogen bonding and solvent accessibility.[11,12] This method of stable-isotope introduction, termed hydrogen exchange (HX), can be studied with high-resolution multidimensional NMR[13−15] or mass spectrometry.[16−18] HX methods have been widely used to characterize, among other things, protein unfolding, protein dynamics, ligand binding, and the formation of protein complexes (for reviews, see refs 13 and 19−21). Current limitations in NMR technology generally prohibit HX studies in large proteins ($>40−70$ kDa) or multiprotein complexes and are restricted to relatively pure proteins at millimolar concentrations. With the ability of HX MS techniques to measure HX rates in

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any size protein with relatively small quantities of material, the structural properties and dynamics of large proteins are being explored (i.e., refs 22−25).

Following HX, the amount of deuterium incorporated into a protein can be measured directly with high-resolution MS. To obtain localized information, a protein that has already undergone HX can be digested with pepsin and the deuterium levels in the resulting peptides measured.[26] After the sequence of each peptic peptide is identified, the extent of deuterium exchange into each peptide can be correlated with a specific region of the protein. If tertiary structure information is available for the protein, HX can be related to structural features.

HX MS analyses of large proteins and complex protein mixtures require identification of many peptides (>100 peptides may be produced during digestion of a 60-kDa protein). Rapid identification of the peptic peptides and the ability to distinguish peptides from the protein of interest from other peptides in the digest would substantially speed up the HX MS characterization of proteins and large protein complexes. In this work, we demonstrate the use of stable-isotope-labeled (SIL) amino acids to address these issues. By incorporating a particular type of SIL amino acid into a protein during synthesis, partial amino acid compositions of the peptic peptides can be obtained, thereby allowing their rapid identification without the need for high mass accuracy or MS/MS sequencing. We show that the same HX information can be obtained from SIL proteins as from non-SIL proteins. Finally, we demonstrate that stable-isotope labeling of proteins allows labeled peptides to be easily distinguished from other peptides in a mixture, thereby permitting the HX analysis of a specific protein in a protein complex or heterogeneous mixture.

MATERIALS AND METHODS

Protein Preparation. 6xHis-tagged human ubiquitin conjugating enzyme 9 (UBC9; as in ref 27) was overexpressed in *Escherichia coli* BL21(DE3)pLysS. The 6xHis tag in UBC9 modified the N-terminal sequence to MGHHHHHHSGIAL. Protein expression was induced by the addition of 1 mM IPTG, and the cultures were grown at 30 °C for 4 h. Unlabeled proteins were obtained from cells grown in LB media. To prepare SIL proteins, M-9 minimal medium was supplemented with 200 mg/L each of the 19 unlabeled amino acids. The 20th amino acid containing the stable-isotope label was added at a 50:50 (w/w) ratio of unlabeled/SIL amino acid for a combined final concentration of 200 mg/L. The labeled amino acids used were as follows: [2,2-d_2]glycine (Gly-d_2), [methyl-d_3]methionine (Met-d_3), [3,3-d_2]-tyrosine (Tyr-d_2), [5,5,5-d_3]leucine (Leu-d_3), and [4,4,5,5-d_4]lysine (Lys-d_4) (Cambridge Isotope Laboratories). After overexpression, the bacteria were lysed with sonication and the His-tagged protein was purified with Ni−NTA resin (Qiagen). Electrospray ionization

mass spectrometry was used to verify the purity (>95%), correct average mass (18 886.8 Da), and incorporation of the stable isotopes. Due to the N-terminal His-tag sequence, ∼30% of UBC9 molecules were phosphogluconoylated (see ref 28) but this modification did not interfere with further analysis.

Hydrogen Exchange. Purified proteins were switched from the Ni−NTA elution buffer into 5 mM phosphate buffer/H_2O, pH 7.00, with HiTrap desalting columns (Pharmacia) and concentrated with Centricon-10 molecular weight cutoff membranes (Millipore) to a final concentration of ∼400 pmol/μL, as determined by a Bradford assay. Hydrogen exchange experiments were performed as previously described.[21] Approximately 3 nmol of UBC9 was equilibrated for 30 min at 22 °C in 5 mM phosphate buffer/H_2O, pH 7.00. Hydrogen exchange was initiated by a 15-fold dilution of the sample with 5 mM phosphate buffer/D_2O, pD 7.40. Exchange was allowed to proceed and quenched at defined time intervals by transferring a 25-μL aliquot to a tube containing 25 μL of 100 mM phosphate buffer/H_2O, pH 2.50. The quenched samples were immediately frozen on dry ice and conserved at −80 °C until further analysis. The final volume of 50 μL contained ∼500 pmol of protein.

Analysis of Deuterium Incorporation. Each sample was rapidly thawed on ice and digested with pepsin (1:1 w/w ratio) for 5 min at 0 °C. The resulting peptic peptides were injected onto a 10-cm, 0.254-mm-i.d. column filled with POROS 10-R2 media (see ref 21). The injector, column, and associated tubing were cooled to 0 °C to minimize back-exchange at backbone amide linkages.[29] During HPLC, deuterium that had exchanged into labile sites within side chains reverted back to hydrogen because the exchange rate for these positions is much faster than that of amide linkages.[30] Separation of the peptides occurred in 5 min with a 5−60% acetonitrile gradient and a flow rate of 40 μL/min. Mass analysis was performed with a Finnigan LCQ-Deca ion trap mass spectrometer. The average amount of back-exchange under the conditions used was ∼15%. Measurements were not corrected for back-exchange (as in ref 26) but were obtained under identical conditions and are therefore described as relative deuterium levels rather than absolute deuterium levels. The amount of deuterium incorporation at any given time point was determined by subtracting the centroid mass value of the 0% control (undeuterated sample) from the centroid mass value of the isotopic distribution at a given exchange-in time of a given peak (as described in refs 17, 18, 21, 26, and 31). This procedure effectively removes the contribution of the natural abundance of isotopes to the deuterium distribution at each deuterium exchange-in point.[26] The error of determining the amount of deuterium incorporated with this centroiding approach is only a minor fraction of the overall experimental error for each time point (∼±0.20 Da).

HX in Bacterial Lysate. *E. coli* [strain BL21(DE3)pLysS] were grown for 4 h at 30 °C, lysed by sonication (lysis buffer: 20 mM Tris-HCl, 500 mM NaCl, pH 7.00), and the cellular debris removed

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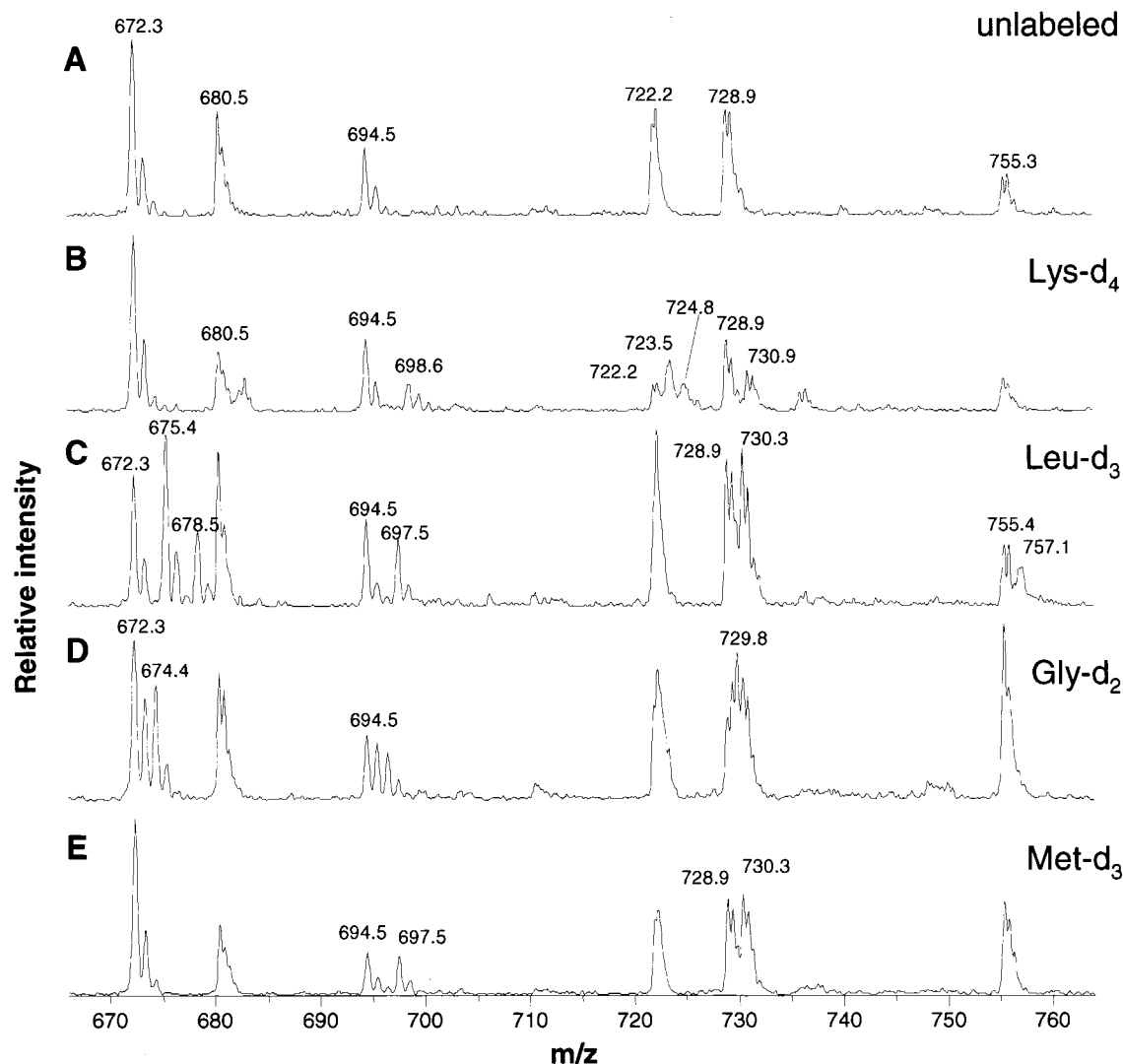
Figure 1. Mass spectra of peptides from unlabeled or SIL UBC9. Peptic digestions of unlabeled UBC9 (no stable-isotope incorporation) (A) and UBC9 grown separately in the presence of four different types of SIL amino acids (B–E) are shown. The digestion conditions were identical for all samples.

by centrifugation. The supernatant, containing cytosolic proteins, was filtered with a sterile 0.20 μM syringe filter (Millipore) and maintained at 4 °C. UBC9 labeled with Lys-d_4 was prepared, purified, and exchanged into 5 mM phosphate buffer/H_2O, pH 7.00 (as above). Equal volumes of filtered lysate and Lys-d_4 UBC9 were mixed for a final concentration of 28 μg/μL total lysate proteins and 7.6 μg/μL Lys-d_4 UBC9 in a volume of 50 μL. The pH of the mixture was 7.00. HX MS analysis was carried out as above. The total amount of Lys-d_4 UBC9 used for digestion and ultimately injected was ~400 pmol/time point.

RESULTS AND DISCUSSION
Stable-Isotope Labeling and Peptic Fragment Identification. To analyze HX with MS, exchange is typically performed at neutral pH and the exchange reaction quenched by reducing the pH to 2.5 and the temperature to 0 °C.[21,26] Under these quench conditions, the rate of HX is reduced enough to allow adequate time to perform the analysis without substantially additional incorporation or loss of deuterium. Quench conditions require that proteolytic digestion of the hydrogen-exchanged proteins be performed with an acid protease. Pepsin is the enzyme of choice,

and although its cleavage pattern cannot be predicted, it cuts reproducibly under the same conditions. Therefore, the sequence of each pepsin fragment must be determined.

To facilitate rapid identification of pepsin fragments, SIL amino acids were used to provide partial amino acid composition information for each peptide. Proteins were expressed and SIL in *E. coli* in a scheme similar to the isotopic labeling of proteins for NMR analysis where bacteria are grown in minimal media containing a specific SIL amino acid.[9] The SIL amino acids chosen for these studies contained 2–4 deuterium atoms covalently bonded to carbon, positions that do not undergo hydrogen exchange. A mixture of labeled and unlabeled amino acids in an approximately 1:1 molar ratio was added to the media. As a result, peptides containing a SIL amino acid have both their normal isotope distribution reflecting the natural occurrence of isotopes and an additional isotope distribution resulting from additional deuterium atoms in the labeled amino acid.[9]

Figure 1 shows electrospray mass spectra for a group of peptides from pepsin digestions of unlabeled UBC9 (Figure 1A) and UBC9 expressed in media containing various SIL amino acids

Table 1. Potential Peptides Found in Mass Searches of UBC9 Identified with Stable-Isotope Composition Information

searched mass[a]	composition[b]				peptides matching searched mass		
	K	L	G	M	coord	sequence	mass[a,c]
1455.8 (728.9^{+2})	1	1	1	1			
	1	1	1	1	32−45	VAVPTKNPDGTMNL[d]	1455.74
	2	1	0	0	81−92	KCKFEPPLFHPN	1455.73
	0	1	0	0	84−95	FEPPLFHPNVYP	1455.72
1508.6 (755.3^{+2})	0	1	0	0			
	0	2	1	0	93−106	VYPSGTVCLSILEE	1508.74
	1	2	1	0	110−122	WRPAITIKQILLG	1507.92
	0	1	0	0	127−140	LNEPNIQDPAQAEA[d]	1508.71
2163.6 (722.2^{+3})	2	0	0	0			
	0	3	1	0	121−140	LGIQELLNEPNIQDPAQAEA	2163.37
	0	2	0	0	126−144	LLNEPNIQDPAQAEAYTIY	2163.36
	3	0	0	0	148−165	RVEYEKRVRAQAKKFAPS[d]	2163.51
	2	1	3	2	39−58	PDGTMNLMNWECAIPGKKGT	2163.52
	2	1	3	2	40−59	DGTMNLMNWECAIPGKKGTP	2163.52
693.5 (694.5^{+1})	1	1	?	1			
	1	1	0	1	65−69	FKLRM[d]	693.40
	1	1	0	1	68−72	RMLFK	693.40
	1	0	0	0	151−155	YEKRV	693.38

[a] Monoisotopic for mass <2000, average for mass >2000. [b] Partial amino acid composition. The first line for each peptide is the composition determined from mass spectra shown in Figure 1 while the remaining lines are the compositions of the peptides matching the searched mass. Amino acids are abbreviated according to the one-letter code: K = lysine, L = leucine, G = glycine, M = M. [c] Theoretical, calculated mass of peptide. [d] Confirmed peptide sequence.

(Figure 1B−E). The spectrum in Figure 1A shows peptides containing the natural abundance of isotopes. The spectra in Figure 1B−E show additional peaks as a result of stable-isotope labeling. The mass spectra of the peptide at m/z 694.5 illustrates how partial amino acid composition information can be obtained. When UBC9 bearing 50% Lys-d_4 was digested (Figure 1B), a second isotope distribution was observed at m/z 698.6, shifted 4 Da higher than the natural distribution. Similarly, for the same peptide, a second distribution appeared 3 Da higher in both Leu-d_3-labeled protein (Figure 1C) and Met-d_3-labeled protein (Figure 1E). The second, higher-mass distribution indicates that the peptide contains the indicated SIL amino acid in its sequence. Because the relative amount of the SIL amino acid is ~50%, the distributions have approximately the same intensity. The results in Figure 1B−E reveal that the peptide at m/z 694.5 contains one lysine, one leucine, and one methionine. The doubly charged ion at m/z 728.9 also contains one lysine, one leucine, and one methionine. In contrast, the peptide at m/z 680.5 contains one lysine but no leucine, methionine, or glycine. Thus, characteristic isotope patterns resulting from incorporation of different SIL amino acids allow determination of partial amino acid composition.

With both mass and partial composition information, the protein sequence was interrogated to identify peptides with the correct mass and composition (see also ref 10). Table 1 shows the results of such a search for four ions in Figure 1. The masses and partial composition are shown in the first line of each section, and the possible peptides with the correct mass and composition are shown below. For the peak at m/z 728.9, there are three possible peptides in UBC9 with this mass. The calculated masses of these three peptides differ from one another by 0.02 daltons, making identification of this peptide based solely on mass impossible at this instrumental resolution (~1500 fwhm). However, the partial amino acid compositions of the potential peptides are different and allow assignment of this peak to the sequence representing amino acids 32−45, the only sequence that matches

the composition information obtained from the mass spectra of the SIL peptides. MS/MS sequencing confirmed the mass assignments and peptide identification (data not shown), validating the use of SIL amino acids in peptic peptide identification.

It is possible, but very rare especially for longer peptides, that several peptides may have the same mass and partial amino acid composition but different sequences. In these cases, MS/MS sequencing is required to differentiate between peptides. This is illustrated for the peak at m/z 694.5 (see Table 1) for which there are two possible sequences with the same partial amino acid composition and the same mass.

As expected, the isotope patterns for peptides from Lys-d_4-, Leu-d_3-, and Met-d_3-labeled UBC9 had the anticipated mass shifts; e.g., a new isotope distribution appeared 4 mass units higher in Lys-d_4-labeled UBC9, etc. Isotope patterns for some peptides from Gly-d_2-labeled protein, however, did not have the anticipated 2-Da mass shift. MS/MS sequencing verified that the peptide at m/z 694.5 did not contain any glycine residues (see also Table 1). Nevertheless, additional peaks on the high-mass side of the isotope pattern for this peptide from Gly-d_2-labeled UBC9 indicated the presence of additional deuterium atoms. Deuterium atoms from Gly-d_2 can become scrambled into other amino acids (serine, methionine, and cysteine) during metabolism in bacteria.[32] For in vivo synthesis of proteins in bacteria, amino acids that are at the end of metabolic pathways are the best for this kind of labeling study because they tend not to scramble their atoms into other amino acids. These include the following: leucine, lysine, methionine, tyrosine, and valine, of which the first four are easily obtainable from commercial sources. With the availability of in vitro translation systems, any labeled amino acid can be incorporated into proteins during synthesis without the scrambling associated with cellular metabolism (see ref 33).

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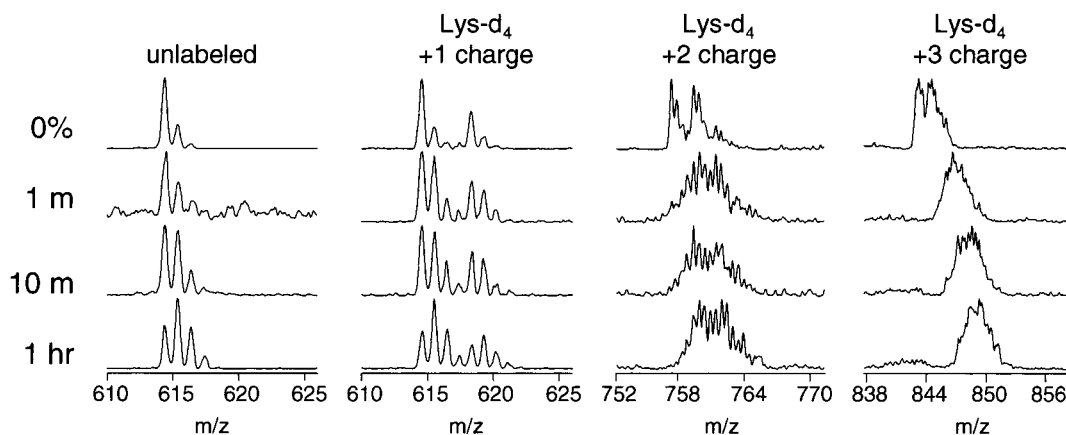(33) Kigawa, T.; Muto, Y.; Yokoyama, S. *J. Biomol. NMR* **1995**, *6*, 129−134.

Figure 2. Mass spectra of representative peptic peptides produced after hydrogen exchange into UBC9. The amount of time each protein spent in D_2O before exchange was quenched is shown on the left with 0% indicating an undeuterated control sample. Unlabeled UBC9 (left panel) contains no SIL amino acids while the remaining peptides were produced from UBC9 labeled with Lys-d_4. Peptides of three different charge states are shown.
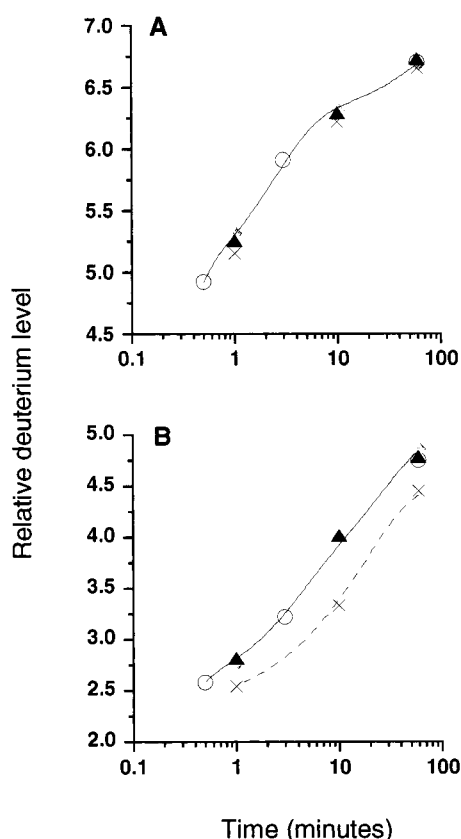

Figure 3. Hydrogen exchange curves for unlabeled or SIL UBC9. The relative amount of deuterium (see text) was determined from the centroid values of the isotopic distributions representing each peptide. Symbols: open circle, unlabeled UBC9; solid triangle, Lys-d_4-labeled UBC9; diamond, Leu-d_3-labeled UBC9; cross, Lys-d_4-labeled UBC9 in bacterial cell lysate. (A) Data for a singly charged peptide m/z 1457 representing the sequence 32−45 of UBC9. (B) Data for a triply charged peptide m/z 843 representing the sequence 11−31 of UBC9. Plots were curve fitted according to ref 21, The dotted line is the curve fit for peptide m/z 843 in the lysate.

Figure 4. A 12% SDS−PAGE gel showing relative protein levels of UBC9 and cell lysate in the mixture used for HX studies. Protein, $\sim$60 μg of Lys-d_4-labeled UBC9, molecular weight 18 900; mix, $\sim$30 μg of Lys-d_4-labeled UBC9 mixed with $\sim$112 μg of total lysate proteins; lysate, *E. coli* cell lysate, $\sim$220 μg of total protein.

SIL techniques are by no means a replacement for the conventional MS/MS techniques that are normally used to sequence and identify peptides. The identification of peptides using stable-isotope-labeling techniques, however, is advantageous for several reasons and may be best utilized in combination with MS/
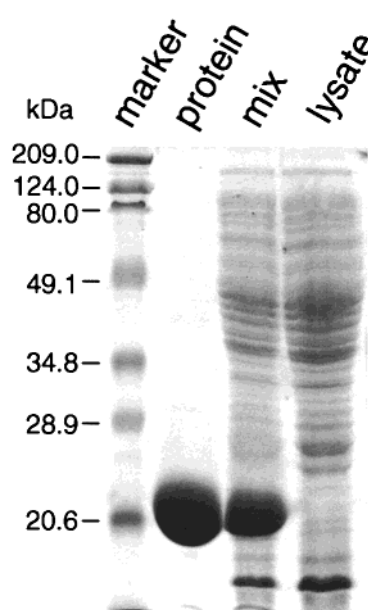
MS. First, SIL helps narrow the field of peptides that must be identified, whether by SIL techniques alone or in combination with MS/MS. In a large protein complex containing one SIL protein, only those peptides that display characteristic isotope patterns indicating the presence of SIL amino acid(s) need to be identified. This approach minimizes the identification of peptides from other unlabeled proteins that are not from the protein of interest. Second, whereas parent ions with signals that are too weak to yield daughter ions in MS/MS spectra cannot be reliably identified, SIL peptides can be identified so long as the signal is intense enough to be observed. Identification of SIL peptides can therefore be made with less material. Finally, SIL techniques can be used to identify many peptides without the additional time required to interpret MS/MS spectra. With isotope labeling, only two experiments (e.g., spectra of digests from protein labeled with either Lys-d_4 or Leu-d_3) should be sufficient for identification of most
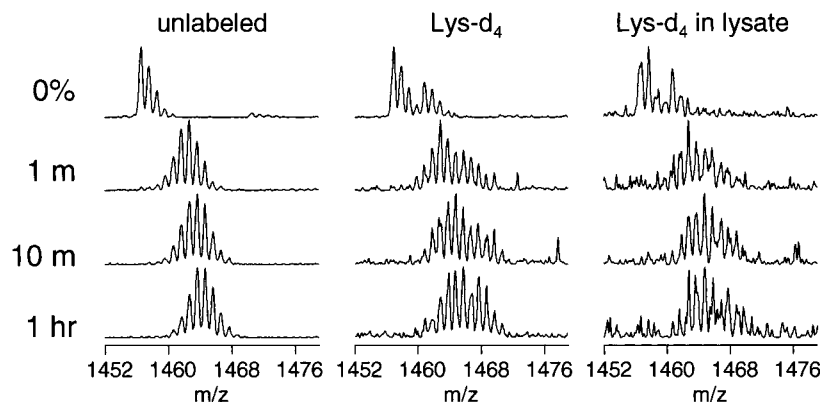
Figure 5. Comparison of mass spectra from HX into free UBC9 or UBC9 in *E. coli* lysate. The amount of time each protein spent in D_2O before HX was quenched is shown on the left with 0% indicating an undeuterated control sample. Unlabeled UBC9 contains no SIL amino acids while the remaining peptides were produced from Lys-d_4-labeled UBC9. Lys-d_4 in lysate is the mixture shown in Figure 4.

peptides. Because lysine and leucine residues together comprise 15% of all amino acids in proteins, statistically at least one of them should be present in most peptides.

Hydrogen Exchange of SIL Proteins. To demonstrate that the incorporation of SIL amino acids has no effect on HX MS measurements, UBC9 was prepared with and without Lys-d_4 or Leu-d_3. In separate experiments under identical conditions, labeled or unlabeled UBC9 was exposed to deuterium for specific periods of time. After quenching and pepsin digestion, mass spectra of the resulting peptides allowed the amount of deuterium incorporated into native UBC9 or SIL UBC9 peptides to be compared. Several representative peptides of different charge states are shown in Figure 2. A peptide from unlabeled UBC9 with m/z 614 (Figure 2, left) exhibited a slow accumulation of deuterium during a 1-h incubation in D_2O buffer. The same peptide from Lys-d_4-labeled UBC9, which had an additional higher-mass isotope distribution corresponding to the incorporation of the Lys-d_4 label, incorporated deuterium at the same rate as the unlabeled peptide of m/z 614. At each time point, the overall shape of the isotope distribution of unlabeled peptide was the same as the lower- or higher-mass isotope distribution in the Lys-d_4-labeled sample. The characteristic isotope patterns illustrated in Figure 2 for peptides from SIL proteins can be used as signatures for the labeled proteins during HX experiments.

The amount of deuterium incorporated at each time point during HX into SIL proteins was determined by subtracting the centroid mass value of the isotope distribution for the undeuterated 0% control from the centroid mass value of the entire isotope distribution of a given peak (as described in refs 17, 18, 21, 26, and 31). The error of determining the centroid value for a given distribution was estimated at ±0.05 Da at this instrument resolution (~1500 fwhm), and the error of each data point was ~±0.20 Da (see also ref 31). Plots of the relative amount of deuterium uptake for two representative peptides are shown in Figure 3. The deuterium uptake in peptides from native UBC9, Lys-d_4-labeled UBC9, and Leu-d_3-labeled UBC9 was found to be the same within experimental error (±0.20 Da). Deuterium levels in other peptides representing >90% of the sequence of UBC9 were also found to be the same for unlabeled or SIL UBC9 (data not shown). These results indicate that incorporation of a SIL amino acid does not change HX behavior. HX into specific regions of a protein can

still be followed in SIL protein and is essentially identical to HX in native unlabeled protein.

Hydrogen Exchange in a Bacterial Cell Lysate. Studying hydrogen exchange of proteins in complexes and in complex mixtures has the potential to reveal information about the structural and dynamic properties of these proteins when part of larger cellular machines. Analyses of this type have previously been extremely difficult. Using our stable-isotope approach, it should be possible to tag individual proteins in mixtures with SIL amino acids and, by observing their characteristic isotope patterns, distinguish them from other proteins in the mixture. Simultaneously, as illustrated above, HX analysis can be performed on such tagged proteins.

To demonstrate the feasibility of this approach, HX was measured for Lys-d_4-labeled UBC9 mixed with a bacterial cell lysate. Lys-d_4-labeled UBC9 was prepared separately and purified. A cell lysate from *E. coli* was also prepared. Purified UBC9 and the cell lysate was mixed (Figure 4) such that the total amount of UBC9 protein (30 μg) was ~25% of the total amount of cytosolic proteins from the lysate (112 μg). A short HX time course was performed, as for the UBC9 proteins alone (see above). The exchange reaction was quenched, and the samples were digested with pepsin in a 1:1 ratio of pepsin/UBC9 protein for 5 min at 0 °C. The digest was injected onto the HPLC column (see Materials and Methods) and desalted twice as long as for UBC9 digests alone before mass spectrometric analysis. Longer desalting prevented high concentrations of small molecules and salts present in the bacterial lysate from entering the mass spectrometer. The injected peptides were separated (as for UBC9 alone) and their masses determined. Digestion of the large number of cytosolic proteins produced many more peptides as well as substantial amounts of partially digested proteins. Complete digestion of all proteins in the cell lysate was not required since UBC9 appeared to be completely digested. Most peptides found in pepsin digests of UBC9 alone were also found in digests of UBC9 in the cell lysate.

While the mass spectra of the UBC9/cell lysate mixture were complex with much higher backgrounds then the spectra of pure UBC9, the characteristic isotope distributions from Lys-d_4-labeled UBC9 peptides were obvious. For example, Figure 5 compares the mass spectra of a peptide during a HX time course for
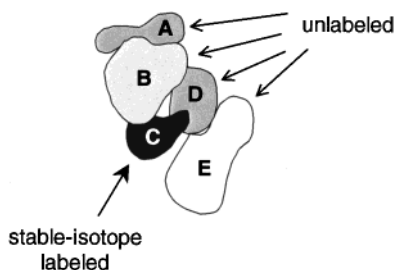
Figure 6. Hypothetical protein complex where SIL proteins can be useful for simplifying hydrogen exchange analysis. One protein (C, black) is SIL while proteins A, B, D, and E are not. The choice of the SIL protein depends on which one is to be studied.

unlabeled UBC9, Lys-d_4-labeled UBC9, and Lys-d_4-labeled UBC9 in the bacterial cell lysate. The peak shape is very similar for the UBC9 Lys-d_4 protein whether alone or in the cell lysate, and it is clear that this peak contains stable isotopes when compared to the peak shape of the unlabeled protein. Other peptides showed similar results (data not shown).

The relative amount of deuterium incorporated into two regions of the UBC9 protein when it was in the cell lysate was determined. As shown in Figure 3, although exchange in one region (panel A, m/z 1457 peptide) had identical deuterium exchange for UBC9 alone or in the cell lysate, that of another region (panel B, m/z 843 peptide) decreased in the presence of the cell lysate. The decrease in HX for the peptide of m/z 843 suggested that this region of UBC9 may bind to or associate with something in the lysate such that HX is reduced in this region. Indeed, the sequence of UBC9 associated with this peak (amino acids 11−31) was shown to be a binding interface for the protein SUMO-1, a component of the *E. coli* cytosol.[27]

These results demonstrate the feasibility of measuring HX in complex mixtures. Since measurement of HX in a bacterial cell lysate is possible, measurement of HX in less complex systems should be comparatively easy. This point is illustrated in Figure 6. For a hypothetical protein complex of five proteins with an average molecular weight of 50 000 each, the identification of peptides from pepsin digestion of the 250 000 complex will be challenging. However, if one of the five proteins is SIL, the peptides arising from this SIL protein during digestion will be distinguishable from other peptides arising from non-SIL proteins in the complex. The analysis and interpretation of peptide HX will be far simpler and time will not be spent identifying and interpreting HX results from peptides that are not in the protein of interest. Complex chromatography steps to separate the proteins in the complex prior to digestion can also be avoided.

CONCLUSIONS

By using SIL proteins, as we have demonstrated, hydrogen exchange of a particular protein in a mixture can be followed. With so many proteins existing as part of multi-protein machines and protein complexes, this methodology may prove helpful for characterizing the details of the folded state or structural changes in proteins within these machines and complexes.